#36 6P1816
Gwdmm 05/14/1998

PATENT
Attorney Docket No. 4249.0002-05

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

John B. SULLIVAN et al.

Serial No. 08/405,454

Filed: March 15, 1995

For: ANTIVENOM COMPOSITION CONTAINING FAB FRAGMENTS (amended)

Group Art Unit: 1816

Examiner: Ron Schwadron, Ph.D.

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

FIRST DECLARATION OF FINDLAY E. RUSSELL, M.D., PH.D.
UNDER 37 C.F.R. § 1.132

I, Findlay E. Russell, M.D., Ph.D., do hereby declare and say as follows:

1. I am a citizen of the United States and am currently a resident of the United States.

2. I received a B.A. from Walla Walla College in 1941.

3. I received an M.D. from Loma Linda University in 1950.

4. I received a Ph.D. from the University of Santa Barbara in 1974.

5. I received an honorary L.L.D. from the University of Santa Barbara in 1978.

6. I am currently Research Professor of Pharmacology and Toxicology at the University of Arizona in Tucson, Arizona.

7. I have held teaching and research positions in several universities and government agencies in the fields of physiology, neurology, pharmacology, toxicology, and toxinology. These positions are listed on my Curriculum Vitae, which is attached as Exhibit 1.

8. I currently hold positions in numerous societies in the fields of physiology, neurology, pharmacology, toxicology, toxinology, and herpetology. These positions are listed on my attached Curriculum Vitae and include the following:

Fellow, Royal Society of Medicine
Fellow, International Society of Toxicology (Pres. 1961-1966);
Fellow, Herpetology, Natural History Museum (London);
Member, American Academy of Clinical Toxicology
(Theines Award, 1965); and
Member, Western Pharmacology Society (Pres. 1972-1973).

9. I have received numerous honors and awards, which are listed on my attached Curriculum Vitae. Many of these honors and awards relate to my work with venoms, and they include the following:

Chairman, Ad Hoc Committee on Snakebite,
National Academy of Medicine, 1962;
Chairman, Ad Hoc Committee on Marine Fish Poisoning
World Health Organization, 1972, 1976;
Member, Ad Hoc Committee on Snake Antivenoms,
World Health Organization, 1969, 1979;
Co-Chairman, National Seminar on Protein Chemistry,
Snake Venom and Hormonal Protein, US-ROC,
National Science Foundation, 1978;
Editor, Toxicology Newsletter, 1955-1960;
Editor, Toxicon, 1962-1970; and
Corresponding member of Academy of Sciences of Yugoslavia, Slovenia.

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10. I have served as a consultant on venoms and venomous animals to the following organizations:

National Academy of Sciences;
American Medical Association;
American Association of Poison Control Centers;
U.S. Armed Forces;
International Red Cross;
National Science Foundation;
National Institutes of Health;
Office of Naval Research;
NASA;
FDA; and
the World Health Organization.

11. I am a coauthor of 370 papers, 26 textbook chapters, and 9 books in the fields of toxicology, toxicology, pharmacology, physiology, and medicine.

12. A substantial part of my 47-year professional career has been directed to research in the field of pharmacology and toxinology, particularly to research involving snake venoms, their *in vivo* effects in humans, and treatments for snake envenomation.

13. I am a co-inventor of U.S. Patent Application No. 08/405,454, filed March 15, 1995 ("the subject patent application").

14. I have read the subject patent application and the June 19, 1997, Office Action (Paper No. 29). I have been asked to provide the following comments regarding this patent application and Office Action.

SNAKE VENOMS

15. A venom is a toxic substance produced by a plant or animal in highly specialized cells or an organ and usually delivered through a biting or stinging act. Although venoms can be simple substances, as in some marine animals, in snakes

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they are often very complicated mixtures of individual toxins, including proteins of large and small molecular weights, phospholipases, hyaluronidase, collagenase, acidocologanase, l-amino acid oxidase, hydrolyses, nucleotideases, lipids, metalloproteins, free amino acids, steroids, aminopolysaccharides, amines, quinones, 5-hydroxytryptophan, and other substances. For example, snake venoms of the family Crotalidae comprise at least 20 different compounds. In some *Crotalus sp.* snake venoms, there may be 100 different protein fractions, 25 of which may be enzymes. Due to their complexity, the full composition of snake venoms is unknown.

16. Not only is the full composition of snake venoms unknown, but the pharmacological effects of some constituent toxins are unknown. Although the individual components of some snake venoms are known to have pharmacologic activities, including hematologic, cardiotoxic, neurotoxic, and other properties, each component may have more than one of these activities, and components may have different actions on different cell types. Furthermore, some of the more important reactions in humans to *Crotalus* envenomation are autopharmacologic or the result of synergisms between different venom components. As a result, of the at least 100 known components of *Crotalus* snake venoms, less than 20 compounds have known pharmacologic activities. Russell, F.E. (1980) *Snake Venom Poisoning* at p. 139 (attached as Exhibit 2).

17. Indeed, it would have been clear to a researcher in the field that we used the term "venom" in the subject patent application to mean a venom comprising several different toxins, not just a single toxin. Each venom discussed in the application

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contains several toxins. Furthermore, the application specifically discusses isolating specific venom proteins (toxins) from the snake venom. Specification at 6, last sentence. Accordingly, a researcher in the field would have understood from the subject patent application that we used the term "venom" in the subject patent application to mean a mixture of toxins, not a single toxin isolated from a venom.

18. The term "antivenin" was first used to identify the first antiserum for snake venom poisoning, and we specifically defined "antivenin" in the specification in this way:

> Antivenin is a suspension of venom-neutralizing antibodies prepared from the serum of animals (typically horses) hyperimmunized against a specific venom or venoms.

Specification at 4, lines 19-22. Although the terms "antivenin" and "antivenom" are often interchanged, researchers in the field now use the term "antivenom" because the World Health Organization ("WHO") has decided that "antivenom" is the preferred term. WHO/B5/80-1292 BLG/VEN/80.1 Rev. 1 (attached as Exhibit 3). Indeed, although I was one of the dissenters in the WHO vote and prefer the term "antivenin," I have previously indicated that the terms "antivenin" and "antivenom" are now interchangeable: "ANTIVENIN (ANTIVENENE, ANAVENIN, ANTIVENIMEUX, ANTIVENINIUM, ANTIVENOM) . . . ." Russell, F.E. (1988) Snake Venom Immunology: Historical and Practical Considerations. *J. Toxicol.-Toxin Rev.* 7(1), 1 (attached as Exhibit 4).

19. At the time of the application, the only commercially available antivenom for envenomation by North American snakes of the family Crotalidae was Antivenin (Crotalidae) Polyvalent (equine origin) (Wyeth Laboratories, Philadelphia, PA). Since

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this was the only commercially available antiserum for snakes in the United States, it was sometimes referred to as simply "antivenin." However, it would have been clear to a researcher in the field that our recitation of "antivenin" in the specification referred generically to all antivenins, not specifically to Wyeth's Antivenin (Crotalidae) Polyvalent (equine origin) because, among other reasons, we specifically referred to Wyeth's product as "ACP" in the application. E.g., specification at 2, line 11. Furthermore, we compared Wyeth's product to our claimed antivenom in the specification at page 23, lines 5-15.

Prior Treatment of Crotalidae Envenomation

20. Approximately 8,000 people are bitten by venomous snakes in the United States each year. Most of these people are bitten by *Crotalus*, a genus of the family Crotalidae. Before the advent of pharmacological methods of treating snake envenomation, it is estimated that envenomation by *Crotalus* resulted in approximately 7% mortality.

21. The most effective and most common treatment of Crotalidae envenomation is the administration of antivenom. The first reported use of a snake antivenom in humans occurred in the late 19th century. The only commercially available antivenom for North American Crotalidae is ACP, which first became available in 1947. Soon after the development of the first antivenoms, doctors recognized that they could elicit serum sickness, an allergic reaction to the antisera that was sometimes more deleterious than the venom. Over 75% of patients treated with ACP develop some manifestation of serum sickness. The problem of serum sickness can be so great

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that physicians may not administer antivenom for some cases of envenomation. Indeed, ACP can only be obtained in a kit that also contains test serum for possibly detecting serum sickness before administering the antivenom.

22. The serious deficit of serum sickness with antivenom has long contributed to extensive research on modifying existing antivenoms or developing new antisera. Since the serum sickness results from immune reactions of the patient to the immunoglobulin component of the antivenom, which actually binds to the venom toxins, much of this research focused on using fragments of immunoglobulin molecules that might not provoke a immune reaction.

23. As the figure attached as Exhibit 5 shows, a molecule of immunoglobulin comprises two heavy chains and two light chains with the heavy chains linked by two disulfide bonds at their hinge region. Each light chain forms an antigen binding site with the corresponding heavy chain at the end distant to the hinge region.

24. Cleavage of immunoglobulin with pepsin cleaves the IgG molecule below the disulfide bonds, resulting in a single Fc fragment and a single $F(ab)_2$ fragment (sometimes called an $F(ab)'_2$ fragment), which contains the two antigen binding sites. In contrast, cleavage of IgG with papain cleaves the immunoglobulin molecule above the disulfide bonds, resulting in a single, larger Fc molecule and two Fab fragments (sometimes called F(ab) fragments), each containing a single antigen binding site. Specification at 2, lines 25-43.

25. In the 1960s, researchers began experimenting with antivenoms comprising $F(ab)_2$ fragments. These became commercially available outside the U.S.

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in 1969. Although these $F(ab)_2$ antivenoms produced less serum sickness, as would be expected from their greater purity, such antivenoms appeared to some to be less effective than antivenoms comprising whole immunoglobulin. Consequently, Crotalidae antivenoms comprising $F(ab)_2$ fragments were not produced in the United States.

Antivenoms Comprising Fab Fragments Were Expected to Fail

26. As of 1984, no significant improvements in antivenoms had been made since antivenoms comprising $F(ab)_2$ fragments became available in 1969. Significantly, although serum sickness had long been recognized as a major problem with antivenoms, and although smaller antibody fragments had long been known to be less immunogenic, no researcher developed an antivenom comprising the smaller Fab fragments. The development of antivenoms comprising antibody fragments stopped at the larger $F(ab)_2$ fragments because researchers in the field expected that Fab fragments would be less effective than $F(ab)_2$ fragments. Indeed, those researchers believed that Fab fragments might actually alter the toxicity of a Crotalidae venom.

27. Researchers in the field were concerned that antivenoms comprising Fab fragments would be less effective than antivenoms comprising $F(ab)_2$ fragments because: 1) the Fab fragments would not prevent the various venom toxins from binding to their site of action as well as the $F(ab)_2$ fragments; 2) the Fab fragments would not precipitate the various venom toxins; and 3) the Fab fragments would not neutralize sufficient venom toxin before being cleared because of their short half-life.

28. Immunoglobulins neutralize toxins in several ways. For example, they bind specifically to epitopes present on the toxins. In the case of a polyclonal

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antivenom, this may involve several epitopes present on more than one antigen. These antigen-antibody complexes are readily eliminated by the reticuloendothelial system, or by other mechanisms.

29. Since $F(ab)_2$ fragments contain two antigen binding sites, like whole immunoglobulin, it was suspected that they could more effectively bind to repeating antigenic determinants on large proteins than could Fab with only one binding site. Sell, S. (1987) *Basic Immunology: Immune Mechanisms in Health and Disease* at p. 89, Fig. 6-3 (attached as Exhibit 6). As a result, it was felt that while Fab fragments might bind to venom toxins, they would not be as effective as whole IgG or $F(ab)_2$ fragments.

30. Furthermore, researchers in the field expected that antivenoms comprising Fab fragments would not be as effective as antivenoms comprising $F(ab)_2$ fragments because Fab fragments have a shorter half life than $F(ab)_2$ fragments *in vivo*. Venom components are usually injected into subcutaneous tissues. Since many of the venom toxins are large, hydrophobic molecules, they are slowly released from these injection areas. This results in the "venom depot effect" where toxins are continuously released into the systemic circulation long after the initial bite.

31. The molecular weight of an Fab fragment is in the range of 45-55 ~~kd~~ kDa. As can be seen from Exhibit 5, the molecular weight of an $F(ab)_2$ fragment is over twice the molecular weight of an Fab fragment and approximately the same molecular weight as a whole IgG. As a result of these differences in molecular weights, Fab fragments are eliminated more quickly than $F(ab)_2$ fragments and whole IgG. Unbound Fab fragments

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are small enough to be removed by the renal system. Consequently they have a half-life of about 17 hours. Indeed, Fab fragments are completely eliminated in only 24 to 26 hours.

32. In contrast, $F(ab)_2$ fragments and whole IgG are too large to be eliminated by the renal system. Consequently they have a longer half-life, approximately 50 hours. The shorter half-life of Fab fragments compared to the half-life of venom, and compared to the half-life of $F(ab)_2$ fragments, led researchers in the field to expect that antivenins comprising Fab fragments would not be effective against Crotalidae envenomation.

33. Not only did researchers in toxinology and pharmacology believe that antivenoms comprising Fab fragments would be less effective than antivenoms comprising $F(ab)_2$ fragments, they suggested that antivenoms comprising Fab fragments might actually be harmful. They expected that the Fab fragments that did bind venom toxins during their short half-lives might not only fail to precipitate the venom toxins, but could actually redistribute the venom toxins to organs where the toxins might concentrate.

34. The binding of whole IgG, $F(ab)_2$ fragments, and Fab fragments to venom toxins is a dynamic process; even at a state of equilibrium, individual venom toxins are constantly being bound and released. The 45-55 kDa molecular weight of an Fab fragment is close to the upper filtration limit of the kidney. As stated, Fab can be cleared by the renal system, but the higher molecular weight $F(ab)_2$ fragments and

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whole IgG cannot. Whole IgG and $F(ab)_2$ fragments are instead cleared by the reticuloendothelial system and the liver, as are any unbound venom toxins.

35. In addition to allowing Fab fragments to be eliminated by the renal system, resulting in a shorter half-life, the small size of Fab fragments, as compared to $F(ab)_2$ fragments, also allows Fab fragments to be distributed to more parts of the body. Researchers in the field were concerned that this rapid clearance and larger volume of distribution of Fab fragments compared to $F(ab)_2$ fragments would result in a more systemic toxicity than a localized one.

36. Researchers in the field also speculated that the larger volume of distribution of the Fab fragments would allow Fab fragments to bind the venom toxins earlier than $F(ab)_2$ fragments. In addition, Fab fragments might bind venom toxins that $F(ab)_2$ fragments could not reach. As the bound Fab-toxin complex circulated throughout the body, however, Fab fragments could periodically release these toxins in their state of equilibrium. The unbound Fab fragments would be rapidly eliminated by the renal system, which could not eliminate the larger Fab-toxin complexes. Researchers, therefore, were concerned that such venom toxins would be redistributed to other areas of the body, perhaps concentrating in areas of high blood flow, especially the kidneys, heart, nervous system, and lungs. Thus, venom toxins that would have been released slowly from the bite site due to the venom depot effect would be redistributed to these areas of high blood flow. In other words, the Fab fragments would effectively serve as a vehicle, redistributing and concentrating these venom toxins from subcutaneous muscles and fat tissues to the kidneys, heart, nervous system, and

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lungs, which would not have otherwise received a relatively high concentration of these toxins.

37. This concern was not merely a theoretical concern, as was later demonstrated by Faulstich *et al.* Faulstich *et al.* (1988) Strongly Enhanced Toxicity of the Mushroom Toxin ∝-Amanitin by an Amatoxin-Specific Fab or Monoclonal Antibody. *Toxicon* 26, 491(copy attached as Exhibit 7). Faulstich *et al.* conducted a series of studies attempting to treat ∝-amatoxin poisoning with Fab fragments. Alpha-amatoxin is a high molecular weight toxin that is similar to some snake venom toxins. As a high molecular weight toxin, ∝-amatoxin cannot be cleared by the renal system. Rather, like many snake toxins, it is cleared by the liver. Since ∝-amatoxin is concentrated in the liver after oral ingestion, it is primarily toxic to liver cells.

38. Faulstich *et al.* discovered that the Fab fragments did not decrease the toxicity of ∝-amatoxin in mice, but rather increased the toxicity of ∝-amatoxin by a factor of 50. Id. at 497. Furthermore, the Fab fragments resulted in ∝-amatoxin being specifically toxic to kidney cells rather than liver cells. This is exactly what one of ordinary skill in the art would have predicted. The Fab fragments bound the high molecular weight ∝-amatoxin, and then unbound it in their state of equilibrium at sites of high blood flow. This unbinding at sites of high blood flow, especially the kidneys, resulted in the ∝-amatoxin being concentrated in these tissues.

39. Similarly, Balthazar *et al.* conducted research on Fab fragments against digoxin. Balthazar *et al.* (1994) Utilization of Antidrug Antibody Fragments for the Optimization of Intraperitoneal Drug Therapy: Studies Using Digoxin as a Model Drug.

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J. Pharm. Exp. Ther. 268, 734 (attached as Exhibit 8). Digoxin is unlike most Crotalidae venom toxins; it is a very small molecule. Digoxin is small enough that the renal system can clear the Fab-digoxin complex. Since the renal system can filter the Fab-digoxin complex, the Fab did not redistribute and concentrate digoxin, as one of ordinary skill in the art would have predicted. Accordingly, Balthazar *et al.* found that F(ab) fragments effectively treated digoxin toxicity.

40. However, Balthazar *et al.* recognized the potential problems of Fab therapy for large toxins, like ∝-amatoxin and some Crotalidae venom toxins:

> First, the alteration of drug distribution which accompanies antibody drug complexation may result in a **potentiation of drug toxicities** or the development of **new drug toxicities in certain cases** . . . . The risk of **redistributing systemic toxicity**, rather than minimizing systemic toxicity, should be appreciated as a potential outcome of the proposed approach.

Id. at p. 738, paragraph bridging cols. 1 and 2 (emphasis added).

41. Accordingly, researchers in the field were concerned that treatment with an antivenom comprising Fab fragments would be a harmful treatment for high molecular weight toxins, not an advisable treatment, because the Fab fragments would redistribute high molecular weight toxins to areas of high blood flow, creating new toxicities and converting a localized toxicity into a systemic toxicity. Faulstich *et al.* confirmed this concern with a toxin that is of a similar molecular weight as many snake venom toxins.

42. Balthazar *et al.* reinforced this concern by showing that this effect did not occur with a low molecular weight toxin that the renal system could clear as part of an

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Fab-toxin complex. Indeed, despite the effectiveness of their treatment, Balthazar *et al.* specifically discussed their concern that Fab fragments might alter drug toxicities or redistribute systemic toxicities.

43. In sum, prior to our invention, researchers in the field did not have a reasonable expectation of success that an antivenom comprising Fab fragments to Crotalidae venom would be effective. Despite known problems with the only commercially available antisera for Crotalidae envenomation and much research since 1947, no researcher had developed an antivenom comprising Fab fragments. Furthermore, although $F(ab)_2$ fragments had been used in antivenoms since the late 1960s, those of ordinary skill in the art had not progressed beyond $F(ab)_2$ fragments to the smaller Fab fragments.

44. Even though Fab fragments were known to be highly effective in reducing the serum sickness, researchers in the field did not create a Crotalidae antiserum comprising Fab fragments because they were sure such a product would not work. Researchers in the field were concerned that such an antivenom would be ineffective because: 1) the Fab fragments could not stearically hinder the Crotalidae venom toxins from binding to their target; 2) the Fab fragments could not precipitate the venom toxins; and 3) the Fab fragments had too short a half-life *in vivo* to be able to bind some snake venom toxins. Furthermore, researchers in the field were concerned that such an antivenom might actually increase the toxicity of the venom by redistributing the more deleterious toxins.

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The Coulter *et al.* Reference

45. I understand that the Examiner has rejected the pending claims in Paper No. 29 over numerous references, including the Coulter *et al.* reference (Coulter *et al.* (1983) Simplified Preparation of Rabbit Fab Fragments. *J. Immun. Meth.* 59, 199 (attached as Exhibit 9)). For the above reasons, none of the references the Examiner has cited, alone or in combination, teach or suggest an antivenom comprising Fab fragments against a Crotalidae venom ~~with~~ would have a reasonable expectation of success.

46. However, the Coulter *et al.* reference merits individual mention in order to clarify the Examiner's understanding of it. Coulter *et al.* used textilotoxin, "a neurotoxin" and the primary toxin in the venom of the Australian brown snake (*Pseudonaja textilis*). Id. at 199, last sentence. The pending claims recite a snake of the genus *Crotalus*, a genus of the family Crotalidae. As can be seen from its name, the snake Coulter *et al.* used is not a member of the genus *Crotalus*, nor even of the family Crotalidae. Rather, it is a member of the genus *Pseudonaja*. Indeed, Cassarett and O'Doul's Toxicology teaches that Coulter *et al.*'s snake is an elapid, Russell (1996) Toxic Effects of Animal Toxins. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, (5th Ed.) at p. 802 (attached as Exhibit 10), and the elapids are of the family Elapidae, not Crotalidae. *Snake Venom Poisoning* at p. 5.

47. Furthermore, textilotoxin is simply a single toxin from Australian brown snake venom. As I discussed above, the terms "antivenom" and "antivenin" mean an immunotherapy mixture against a snake venom, not simply a single snake toxin. As I have also noted, snake venoms are complex mixtures of many substances, including

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many different toxins. Snake venoms, particularly those of snakes of the family Crotalidae, are composed of many different toxins. Each of the individual toxins can act synergistically *in vivo* and may also induce autopharmacologic reactions. Indeed, basic toxicology texts caution against extrapolating results from individual venom toxins to whole venoms. Toxic Effects of Animal Toxins at p. 802; *Snake Venom Poisoning* at p. 168. Accordingly, one would not have expected Coulter *et al.*'s results with Fab to a single **toxin** to predict similar results with Fab to a Crotalidae snake **venom**, including a *Crotalus* snake **venom**.

48. Most importantly, Coulter *et al.* did not treat envenomation with their Fab fragments. Rather, Coulter *et al.* first mixed textilotoxin with their Fab fragments *in vitro*. Coulter *et al.* at p. 901, 3rd full paragraph. Coulter *et al.* then injected the already bound Fab-textilotoxin complex intravenously. This treatment with Fab fragments resulted in neutralization that was essentially equivalent to the treatment with the IgG fragments, just as one would have expected. Since the Fab-textilotoxin mixture was injected intravenously, the Fab did not have the opportunity to redistribute and concentrate the textilotoxin in high blood flow parts. Accordingly, the Coulter *et al.* reference would not have provided a reasonable expectation of success for an antivenom comprising Fab fragments to any venom toxins.

49. Similarly the observation of Coulter *et al.* that enzyme-linked immunoabsorbent assays with higher sensitivity had been claimed when Fab is used instead of whole IgG would not have suggested combining any of the cited references with a reasonable expectation of success. As in the case of Coulter *et al.*'s actual

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results, *in vitro* observation would have been irrelevant to the lack of expectation of success *in vivo* since the reasons one would not have had a reasonable expectation of success were due to the expected *in vivo* action of the Fab fragments.

50. Indeed, Sorkine *et al.* conducted a similar experiment in 1983 by mixing Fab fragments with a venom of a non-Crotalidae snake prior to injection into a mouse, and they obtained similar results. Sorkine *et al.* (1995) Comparison of $F(ab')_2$ and Fab Efficiency on Plasma Extravasation Induced *Viper aspis* Venom. *Toxicon* 33, 257 (attached as Exhibit 11). This treatment resulted in a considerable reduction in capillary permeability. However, the Fab fragments were much less effective when they were administered *in vivo* separately from the venom. As Sorkine *et al.* state "these data showed firstly that the *in vitro* neutralization of the venom by immunoglobulin fragments does not reflect their *in vivo* efficiency." Id. Thus, the Sorkine *et al.* reference shows that one would not have expected Coulter *et al.*'s *in vitro* neutralization results to predict the effectiveness of ~~antivenins~~ antivenoms comprising Fab fragments *in vivo*.

51. In 1984, no clinician or researcher expected that a Crotalidae snake antivenom comprising Fab fragments would be effective in treating Crotalidae snake envenomation. Thirty-seven years of research primarily aimed at reducing the serum sickness produced by ACP and fifteen years of research since the first, disappointing $F(ab)_2$ antivenom had convinced those of ordinary skill in the art that a Crotalidae antivenom comprising Fab fragments would be less effective than the known antivenoms. Furthermore, those of ordinary skill in the art actually expected a Crotalidae antivenom comprising Fab fragments to increase the lethality of the snake

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venom. Accordingly, there was no expectation of success in using any Crotalidae antivenom comprising Fab fragments to treat Crotalidae envenomation, let alone an expectation of success in using a *Crotalus* antivenom comprising Fab fragments to treat *Crotalus* envenomation.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and, further, that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine, imprisonment, or fine and imprisonment under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of this application or any patent issuing therefrom.

Date: April 30, 1998

By: Findlay E. Russell, M.D., Ph.D.
Findlay E. Russell, M.D., Ph.D.

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CURRICULUM VITAE

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Ph.D., University of Santa Barbara, 1974 (1987)
LL.D. (hon.) University of Santa Barbara, 1989

Appointments:

Internship, White Memorial Hospital, Los Angeles, 1950-51.
Giannini Honor Fellow, California Institute of Technology, 1951-52.
USPHS Honor Fellow, California Institute of Technology, 1952-53.
Chief Physiologist, Institute of Medical Research, Huntington Memorial Hospital, Pasadena, California, 1953-55.
Director of Labroatory of Neurological Research, and Venom Poisoning Center, Los Angeles County-University of Southern California Medical Center, 1955-1980.
Assistant Professor (Neurophysiology), Loma Linda University, 1955-58.
Associate Professor (Neurophysiology), Loma Linda University, 1958-61.
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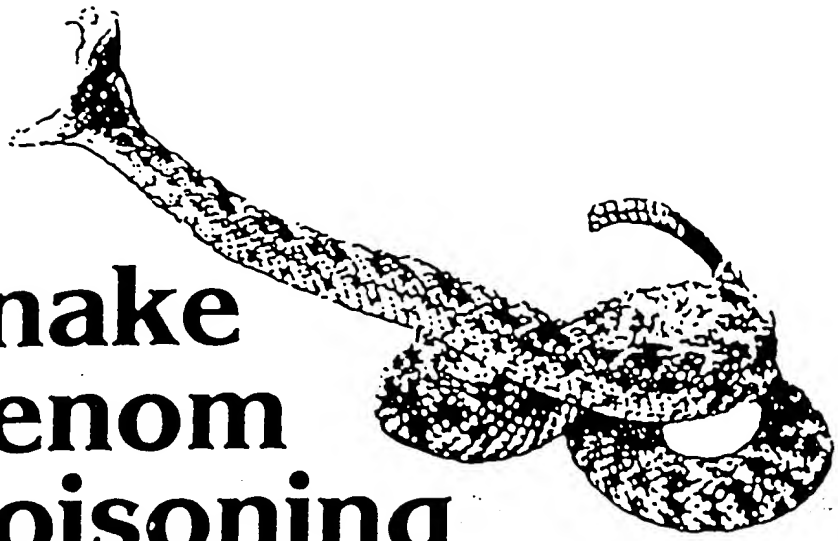
In: *American Men of Medicine*
American Men of Science
Dictionary of International Biography
Leaders in American Science
Notable Americans
World's Who's Who in Science
Who's Who in the West
Who's Who
Two Thousand Notable Americans



snake venom poisoning

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J. B. Lippincott Company
Philadelphia • Toronto

ISBN 0-397-50472-1

Library of Congress Catalog Card Number 79-21661

Printed in the United States of America

6 5 4 3 2 1

Library of Congress Cataloging in Publication Data
Russell, Findlay E
Snake venom poisoning.

Bibliography
Includes index.
1. Snake venom—Toxicology. 2. Poisonous snakes—North America. 3. Serpents (in religion, folk-lore, etc.) I. Title.
RA1242.S53R87 615.9'42 79-21661
ISBN 0-397-50472-1

spider, or fish, is the standard of reference that determines the application of the scientific name. The type of a nominal species is a specific specimen, that of a nominal genus is a nominal species, and that of a nominal family is a nominal genus. The type of a taxon is not subject to change except by exercise of the International Commission on Zoological Nomenclature.

The word snakebite is commonly employed to denote bites by the venomous snakes. Perhaps it is more proper to limit this term to bites by all snakes, whether they are venomous or not. Unfortunately, the words snakebite and snake venom poisoning are so commonly interchanged in both the medical and lay literature that misunderstandings have arisen, as well as errors in clinical judgment. A bite by a venomous snake can, of course, be called a snakebite, but it is more properly called a venomous snake bite. A bite by a venomous snake, in which envenomation has not occurred, as is so often the case, might be termed venomous snake bite without envenomation. But a bite by a venomous snake, in which poisoning has occurred, should be identified as snake venom poisoning, which thus separates it as a distinct entity.

The words legitimate and illegitimate are often used to differentiate a bite that occurs as a natural event in the wild, or in "persons who have no intention of indulging in so unnecessary a risk,"[4] from a bite that occurs in a person who, for one reason or another, is handling a venomous snake. In our own practice, probably 40 per cent of all bites by venomous snakes that we have treated fall into the latter group. This, however, would certainly not be the average for most physicians.

The term exotic snake is used by herpetologists to denote a non-native United States species, "a snake foreign to this country." There are few snakes more exotic than the rattlesnake, but the term is still applied only to foreign snakes. The term exotic antivenins is sometimes used to identify antivenins for non-United States species. However, there is nothing particularly exotic about these antivenins.

Some snakes spit or spray their venom and are called spitting snakes. There are several such species; *Naja nigricollis* and *Hemachatus haemachatus* are the most well known. Spitting is an act of venom discharge unique to some elapids, which spit or spray their venom as part of a defensive act (see pp. 353–361).

Venomous snakes are usually identified by family: Colubridae, Elapidae, Hydrophiidae, Viperidae, or Crotalidae. By the rules of nomenclature, the family name is written in lower case, and the first letter is capitalized. The next category below family level is the genus. The genus name is written in italics, and the first letter is capitalized. The next level below the genus is the species, which is also italicized, and the first letter is lower case. Some species have subspecies, and their names are also written in italics, and the first letter is lower case. Common names are written in lower case, as is the custom in most texts of biology. In some medical texts they appear in italics, but this practice should be avoided.

6

venoms

Upon the first sight I could discover nothing but a parcel of small salts nimbly floating in the liquor; but these saline particles were now shot out as it were into crystals of an incredible tenuity and sharpness, with something like knots here and there, from which they seemed to proceed: so that the whole texture did in a manner represent a Spider's web, though infinitely finer, and more minute; and yet withal so rigid were these pellucid *spicula*, or darts, that they remained unaltered upon my glass for several months. [Mead][1]

A venom is a toxic substance produced by a plant or animal in a highly developed secretory organ or group of cells, and one which the animal can deliver during a biting or stinging act. Venoms may be very simple substances, or like snake venom, they may be very complicated mixtures of different substances. The words toxin and venom are often interchanged, although most investigators use the former when describing the whole or crude poison, and the latter for some component part. The words venom and poison are also interchanged (see p. 3).

Function

A venom may have one or several functions in an animal's armament. It may play a role in offense, as in the capture and digestion of food, or may contribute to the animal's defense, as in protecting it against predators or aggressors. It may also serve both functions.

The black widow spider employs its venom to paralyze its prey before extracting hemolymph and body fluids. The venom is not primarily designed to kill the prey, only to immobilize it. Were it to cause early death, removing the hemolymph and body fluids would be made much more difficult, and would seem inconsistent with design and, perhaps, the spider's survival. Most venoms used in offense are associated with the oral pole, the most functional region for their dispense. Defensively designed venoms, on the other hand, are usually associated with the aboral pole, as in the stingrays, or with dermal tissues, as in scorpionfishes and certain other fishes.[2]

Venomous snakes are considered to be the most highly developed of all the reptiles. This supposition is based on their success in both kinds and

SPECIFIC CHEMISTRY AND PHARMACOLOGY

It is one of the unfortunate facts in the study of the chemistry and physiopharmacology of snake venom that the structure and design are most easily studied by taking the venom apart. This has two shortcomings: Firstly, a destructive process must be used in an attempt to understand a progressive and integrative one; and secondly, the essential quality of the whole venom is destroyed before we have made suitable acquaintance. Often times the process of examination becomes so exacting that the end is lost sight of in our preoccupation with the means, so much so that in some cases the means becomes a substitute for the end.

Animal venoms are complex mixtures; many are composed of proteins of both large and small molecular weights, while others are rich in lipids, steroids, aminopolysaccharides, amines, quinones, 5-HT, or other substances. The composition of most venoms, however, remains unknown. Some snake venoms, particularly those of the crotalids, are composed of as many as 20 different components, while the venoms of elapids appear to be less complex.

Our knowledge of the physiopharmacologic properties of venoms is relatively meager. We know some of the pharmacologic activities of a number of the crude venoms, as well as a few of the properties of a relatively small number of venom fractions, but data on the various specific effects, and the autopharmacologic, synergistic, and antagonistic properties of these complex substances is far from complete.

Perhaps no other area of toxinology has undergone such a complex evolution—both in reality and in the literature—as has the chemistry of snake venoms. I have witnessed in my own life-span an almost complete contraposition, from one that considered snake venoms solely as enzymes, and classified them on this basis, to the recent emphasis on peptide structure, and classifying them accordingly. For the present it seems wisest to consider all snake venoms as complex mixtures containing peptides or polypeptides, enzymes, glycoproteins, and other substances capable of producing several or many pharmacologic activities, some of which are deleterious to living organisms. For the physician it bears repeating that a snake venom should never be considered solely as a "neurotoxin," "cardiotoxin," "myotoxin," or any of the other dozens of loosely articulated synonyms, while dismissing the overall biological activities of the whole venom. In my opinion, the facts are not sufficiently valid to even classify specific components under these broad categories. There are some components that may well have an effect at some specific site or membrane in the nervous system, and nowhere else, but this has not yet been demonstrated to my satisfaction. The basis for calling a specific fraction a "neurotoxin" is usually founded on its action on a particular preparation the investigator has selected; and rarely are the other possible pharmacologic properties or tissue modulations studied in detail. Such findings as those of Harris,[118,119] as well as our own group,[101,120] indicated the

ORGANISATION MONDIALE DE LA SANTE

ENGLISH ONLY

WHO COORDINATION MEETING ON VENOMS AND ANTIVENOMS

A WHO informal meeting took place at the Chemika Zurich from 24 to 27 September 1979. The purpose of the meeting was to coordinate the work in progress throughout the world on the use and standardization of venoms and antivenoms. The list of the participants is shown in Annex I and the agenda in Annex II.

For many years WHO has had an interest in the treatment of bites and stings from poisonous creatures and although there have been informal meetings from time to time none has specifically attempted to collect the data of the clinical effects of snake and scorpion bites and stings and the experiences in their treatment. Furthermore it was recognized that there is an urgent need to correlate such experiences with the laboratory tests being applied to the antivenoms in attempts to measure the potency of these materials. One important advance that could be made in such standardization is the availability of venoms that had been fully characterized and the establishment of international standard antivenoms.

WHO has taken the first step in designating the Liverpool School of Tropical Diseases as the WHO Collaborative Centre for the Control of Antivenoms. As Director of this Centre, Dr H. Alistair Reid agreed to be Chairman of the meeting.

The meeting agreed that in English 'venom' and 'antivenom' were the preferred names rather than venin/antivenin or venene/antivenene.

A. EPIDEMIOLOGY

Incidence and mortality of snake bites, scorpion stings and spider bites

Injuries and death due to snake and spider bites as well as scorpion stings occur in most parts of the world, and especially in the tropics where they may represent a major health problem. Unfortunately, knowledge of their epidemiology is fragmentary due mainly to the lack of reliable statistical data.

In the United States of America, approximately 8000 bites by venomous snakes are reported each year. There are about 12 deaths which occur in the untreated, under-treated, or mistreated children or in members of snake-handling cults. Approximately 1000 scorpion stings are reported each year; the last death was in 1968. About 3000 spider bites (usually Latrodectus or Loxosceles sp) occur each year. Marine animal stings range into several hundred thousand each year but deaths are extremely rare.

Scorpion stings are a major health problem in Mexico where there are an estimated 300 000 cases each year, with about 1000 deaths. Scorpion stings are also important in Trinidad and South America. Spider bites are mainly common in South America and Australia.

In Costa Rica, hospital admissions for snake bite have been estimated as 22.4 per 100 000 population per year, with 5 deaths per 100 000 (mostly due to bites by Bothrops atrox). In South America 90% of snake bites are caused by Bothrops species. Mortality has been estimated as 2.4% but may be as high as 8% when no antivenom is given. After rattlesnake bites (Crotalus durissus terrificus) about 74% of the untreated victims die but in patients receiving antivenom, mortality falls to 12%.

CODEN: JTTRD9 7(1) i-ii, 1-94 (1988)
ISSN: 0731-3837

Journal of Toxicology

Toxin Reviews

Volume 7 Number 1 1988

J. TOXICOL.-TOXIN REVIEWS, 7(1), 1-82 (1988)

SNAKE VENOM IMMUNOLOGY: HISTORICAL AND PRACTICAL CONSIDERATIONS

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ABSTRACT

Man has tried to immunize himself against snake venoms and other poisons since the beginnings of history. The scientific study of antivenins began with the work of Henry Sewall in 1887 and has progressed through the present century. Currently, a large number and diversity of monovalent and polyvalent antivenin preparations produced by well-defined protocols are commercially available around the world. These preparations owe much to the pioneering studies of many research workers, but most notably to the work of Sewall, Calmette, Fraser, Brazil, and Noguchi, and more recently to the studies of Boquet and Minton.

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I. INTRODUCTION

Understanding any subject is enhanced by knowledge of its history. The history of snake venom immunology seems no exception. To appreciate it one must view it through those sciences from which it arose -- immunology, bacteriology, virology, allergenic medicine, herpetology, clinical medicine, and toxinology, to note only a few. For that reason, this review will touch upon some of those disciplines in which basic data have contributed in a significant way to our knowledge of snake venom immunology. Obviously, also, some understanding of the chemistry and pharmacology of the snake venoms themselves is essential to venom immunology, as well as a working knowledge of hematology, laboratory medicine, and the skills of treating trauma. It can be seen that snake venom immunology is hardly an entity unto itself but has arisen through multiple biomedical disciplines during the hundred or so years of its course.

Few subjects have stimulated the minds and imagination of man more than the study of snakes and snake venoms. No animal has been more worshipped yet more cast out, more loved yet more despised, more envied yet more caged; and more collected yet more trampled upon than the snake. The essence of the fascination and fear of snakes has lain in their venom. In times past the consequences of bites by venomous snakes were often attributed to forces beyond nature, sometimes to vengeful deities thought to be

embodied in the serpents. To early peoples the effects of snakebites were so surprising and varied, and so violent and sometimes incapacitating, that the snakes and their venoms became shrouded with myth and superstition (1).

II. HISTORY

"It seems," wrote Galen, "that there is nothing more dangerous in life than poisons, and [the bites] of noxious animals" (2). Galen goes on to say that man can avoid these dangers by fleeing them, but as that is not always possible, he may "fall victim to their bite without warning." Like many early Roman and Greek writers, the description of the bites and stings of venomous animals generally portrayed a picture of desperation and frustration. This, in turn, gave rise to a plethora of therapeutic remedies. The origins of most of these early remedies are still obscure. Even the Peri Therion (On Poisonous Animals) by Apollodorus (3), which probably represents the first Greek treatise on poisons, includes many items that appear to be of Near East origins and then passed down through Aristotle, Diocles, Athenaeus, Pliny, Galen, and Sostratus. Of course, the great collection of these remedies for poisoning comes to us through the laborious poems of Nicander in the Therica et Alexipharmaca (4).

The first cures for snake venom poisioning appear to be established in exorcism, a mixture of incantations, chants, laying on of hands, massage, sprinklings, and anointments with various waters, plants, and earths. These were often associated with an elaborate ceremony by a local doctor-priest and appear to have been equally successful or unsuccessful. The practice of exorcism in snake venom poisoning, however, has not been limited by the passing of time. Even today, some form of witchcraft therapy for snakebite is practiced in almost all parts of the world.

The Bibliography of Snake Venoms and Venomous Snakes lists more than 300 "cures" for snakebite that had been suggested up until its publication in 1964 (5). Many of these antidotes are rooted in folklore. Folk medicine, like folklore, transcends both science and education, and tends to derive its remedies from the simple people of each culture, people who in times past lived closest to nature and, supposedly, to nature's secrets. Often they were poorly educated, if at all, and tended to distrust things they did not understand. Such people seek refuge in those things they assume to be inherent in nature, which possibly appear "instinctive" to them. They may create a remedy from an inaccurate observation or a misinterpretation. They may exaggerate an experience, or even a myth, which when repeated to another becomes a "fact". One need only to examine the current scientific literature to see how a falsehood can be promulgated on the unknowing by presumably knowledgeable scientists or physicians. The myth that Antivenin Crotalidae Polyvalent (Wyeth) does not protect against the local tissue effects of crotalid venoms is a good example in this respect.

A. Early Attempts at Immunization

It is difficult to know when man first attempted to experiment with immunizing himself or others. Perhaps Attabus II of Pergamon (c. 170-113 B.C.) and Mithridates VI (120-63 B.C.) of Pontus were the first of the great Greek physicians to dabble with poisons and immunology (6,7). A reader of Nicander, Mithridates acquired a considerable reputation for his readiness and skill in administering poisons as therapeutic agents for a wide variety of diseases. This interest, and perhaps fear in his own art, led him to attempt to concoct a universal antidote, a concoction called Mithridatum that appears to have been employed for almost 1,600 years, or at least through Tudor times (8).

Mithridates is probably equally well-known for his attempt to "immunize" himself by drinking the blood of ducks, which he had been keeping on a ration of one poison or another. Thus, he might be credited with being the first physician to attempt immunization.

It is obvious that the ancients had some idea of the relationship between a disease state and protection against that disease, and it may be that they had a deeper knowledge of this than is generally believed. It is for sure they had observed that individuals who survived a crippling contagious disease seldom contracted the disease again. Those persons whose unpleasant task it was to gather and bury the dead during plagues were often previous victims of that particular disease. Thucydides (390-460? A.D.) (9) notes that while the plague was raging in Athens there would have been no care for the sick and dying if it had not been for those who had recovered from a previous siege of the disease and who served as attendants. Perhaps immunology grew out of these early observations of the Greeks, for we find similar practices during the plagues of the 12th, 13th, and 14th centuries.

Harvey has stated, "Both the Indians and Egyptians immunized themselves by allowing young snakes with a small supply of venom to bite them. Older snakes were used later until full immunity was achieved" (10). I regret that I have not been able to verify this statement, although it certainly is conceivable. Similar statements have been made by a number of writers (see reference 5). According to A. H. Mohammed (personal correspondence, 1978), early Egyptian priests were sometimes bitten by the horned viper (Cerastes cerastes) used in religious rites, but there is no evidence that this was done for immunization purposes. It is not known if these snakes were defanged or milked before being handled, as more recent North American Indian shamans have done (1).

The "Curados de Culebras" Indians of Mexico were said to gain immunity by inoculating themselves with the ground-up teeth of rattlesnakes. Natives of Guiana, Orinoco, the Amazon, and Central Africa, at least by the 19th century, were known to inoculate themselves with snake venoms for immunity (11). It is thought that the reason charmers, priests and witch doctors, among many other native healers, passed their practices down from generation to generation was their belief that by immunizing themselves the immunity could be transferred to their offspring. Further reviews of early attempts at immunization against snake venoms will be found elsewhere (1,11,13,14).

B. Use of Blood and Blood Products During Early Times

The use of blood in the treatment of various disease states was known to the ancients. It was also given for all manners of poisonings, including those caused by venomous animals. The first viable account of the use of various animal bloods in therapeutics was that of Paulus Aegineta, who employed several kinds of blood for ecchymosis about the eyes and as an anti-inflammatory agent in trephining (6). Also, Galen noted the use of blood for various types of poisonings, including those provoked by venomous animals (2). There were numerous reports from the 1400's to the 1600's of the use of animal blood for transfusions in Europe (15-20). In spite of many reported early successes with blood transfusions in the treatment of disease, it became apparent with the work of Denis (21) that infusion of animal blood into a human was fraught with danger, and by the end of the 17th century transfusions were held in some disrepute by most physicians (22).

C. Immunology

It is difficult at best to assign a date for the beginning of any medical discoveries. Edward Jenner is usually credited

with founding the science of immunology in view of his studies on "Cowpox", which might be considered a mutant of smallpox (24). This credit is aptly given, for his work was certainly a milestone in the science of vaccination. Jenner's vaccination technique was a refinement of a technique known as variolation, which had been practiced in Asia for centuries (25-30) and introduced into England by Lady Wortley Montague (28). Jenner's studies on the cowpox vaccine (31) established its effectiveness and led to its replacement of variolation around the world, including the United States (32-39). Although significant contributions to immunology were made on the basis of Jenner's studies, the major extension of his work was not attained until the development of the germ theory of disease and Pasteur's demonstration of methods for producing artificial immunity to such diseases as anthrax, chicken cholera, and rabies (40,41).

It remained for Roux and Yersin in 1888 to demonstrate that immunity could be developed against a toxin by the production of a specific neutralizing antitoxin in the blood of the immunized animal, and that immunity could be transferred to another animal, that is, passively transferred (42). Clinically, this was demonstrated with tetanus antitoxin by Von Behring and Kitasoto in 1890 (43). Ehrlich showed that antitoxins could not only be produced against bacteria, but also against a chemical (44). From these and other works the era of serotherapy emerged. It remained to Metchnikoff (45), however, to suggest that phagocytosis by leukocytes constituted the most important factor in immunity, although this theory gained acceptance only gradually.

D. Snake Venom Immunology

At this point in time, snake venom immunology began to emerge. Almost completely overlooked in the development of our knowledge on antitoxins, and in particular antivenins, has been the work of Henry Sewall (46) (Figure 1). Although Brunton,

FIG. 1. Henry Sewall (1855-1936). Courtesy Ferdinand Hamburger, Jr. Archives, Johns Hopkins University.

Fayrer, Dumeril, Rudolf, Krehl, and Calmette all subsequently appreciated his important contribution, he did not receive the recognition he so well deserved until years later. When Calmette and a group of French scientists honored Johns Hopkins University with a visit some years after Sewall's experiments, Calmette asked to see the laboratory where the physiologist had carried out his important experiments. Apparently, there were some embarrassing moments. In 1907 Calmette wrote "So long ago as the year 1887, it was shown by Sewall in an important paper on "Rattlesnake Venom" that it is possible to render pigeons gradually more resistant to the action of this venom by injecting them with doses at first very small, and certainly incapable of producing serious effects, and then with stronger and stronger doses . . . he succeeded in making them withstand doses ten times greater than the minimal lethal dose" (47).

The German clinician, Ludolf Krehl, wrote "The foundation for all the works which have been done on animal poisons is to be found in the [work] of Sewall, done at Ann Arbor, Michigan in 1887, the great significance of which was not fully realized until some years afterwards" (48).

In essence, Sewall demonstrated that when pigeons were inoculated with a sub-lethal dose of rattlesnake venom followed by injections of increasing doses to levels above that which would have killed the animals initially, the pigeons developed a resistance to the venom without ill effects. The first page of his famous work is shown in Figure 2.

It can be seen that Sewall initiated his studies with the hope that it might form a worthy contribution to the theory of "Prophylaxis". He notes the analogy between the venom of poisonous snakes and the "ptomaines produced under the influence of bacterial organisms." He developed this analogy from reading the papers by Mitchell and Reichert (49-50) and Wolfenden (51). Sewall had previously observed that various poisons in very small

EXPERIMENTS ON THE PREVENTIVE INOCULATION OF RATTLESNAKE VENOM.

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THE following work was undertaken with the hope that it might form a worthy contribution to the theory of Prophylaxis, and the results obtained during the first stage of its progress are put forward at this time because of the impression that, perhaps, at least their practical significance may induce investigators more fortunately situated for the performance of such experiments to take up the same line of observation. I have assumed an analogy between the venom of the poisonous serpent and the ptomaines produced under the influence of bacterial organisms. Both are the outcome of the activity of living protoplasm although chemically widely distinct, the ptomaines belonging to the group of alkaloids, while the active principles of the venom, according to Mitchell and Reichert[1] and to Wolfenden[2] are of proteid nature.

If immunity from the fatal effects of snake-bite can be secured in an animal by means of repeated inoculation with doses of the poison too small to produce ill effects, we may suspect that the same sort of resistance against germ-disease might follow the inoculation of the appropriate ptomaine, provided that it is through the products of their metabolism that bacteria produce their fatal effects. It is not necessary at this time to consider the bearing of the literature on the subject in question, for there can be drawn from it few, if any, unassailable conclusions.

It is a matter of common experience that with the repeated exhibition of various kinds of poisons in therapeutic doses, more and more of the substance must be employed to produce its physiological action, and, finally, ordinarily fatal doses may be given with impunity. And yet there is reason to believe that this resistance may result from either of two opposite conditions impressed upon the living parts of the body, a pathological or a physiological.

In the first case the sum total energy of the protoplasm is diminished; its irritability is lowered as well as its efficiency as a machine. In the second case the total energy of the protoplasm is not diminished

[1] "Researches upon the Venoms of Poisonous Serpents." *Smithsonian Contrib. to Knowledge*, 674, 1886.

[2] This *Journal*, Vol. VII. p. 327.

FIG. 2. The first page of Henry Sewall's report on immunizing pigeons with rattlesnake venom (Journal of Physiology, 8:203, 1887).

or therapeutic doses eventually lost their effectiveness, and that continually increasing doses had to be used to produce the desired effect. He concluded that these "ordinarily fatal doses may [then] be given with impunity" (46).

Sewall employed the venom of the eastern massasauga, Sistrurus catenatus catenatus, injecting the venom diluted in glycerin along the backs of pigeons just under the skin. It is difficult to determine his initiating or final doses since there is no standard by which they can be measured. Nevertheless, he demonstrated, as in one pigeon, that while the initial lethal dose for a pigeon was two-thirds of a drop of venom, about one month later, having received approximately ten increasing doses of venom, the pigeon could tolerate four and one-half drops without ill effects. Among other matters he noted that: (1) the animal's resistance decreased with time in the absence of sustaining doses of venom; (2) some resistance persisted even after five months; (3) in some experiments he found that if the animals were to die it would be during one of two periods: 3 hours or 15-20 hours after injection; (4) signs of acute toxicity were constant, and consisted of paresis then complete paralysis of the legs, tottering gait, and excessive lacrimal secretions. He also observed that venom kept in glycerin gradually deteriorated. Sewall's report is truly a remarkable contribution. It might also be noted that his work antedates the renowned discovery by Von Behring and Kitasato on diphtheria antitoxin.

In 1889, Kaufmann, using the venom of Vipera berus, obtained somewhat similar results (52), although not with the same high titer as had Sewall, and in 1892, working in Saigon, Calmette began his first experiments on cobra venom, reporting that "It was possible by means of successive inoculations with heated venom to confer . . . a certain degree of resistance to doses invariably lethal to the controls (53).

Kanthack performed a number of experiments on cobra venom and blood, among which was a study on the immunization of animals. From his experiments he concluded that "It is therefore impossible to establish an immunity against the bite of a cobra in this manner [immunization]" (54). This frequently quoted statement has often been used to infer that he questioned the efficacy of an immunization program and a difference with Sewall at this point, but a careful reading of his paper indicated that he was studying some of the problems associated with schedules of immunization, and notes that "with the experience gained by these preliminary experiments, three animals were prepared and accustomed to tolerate large doses . . . they can, however, be accustomed to resist large doses" (controls succumbed to the same doses of venom). There is nothing in this paper that would lead one to question Kanthack's belief in the potential of a well-designed immunization program.

Kanthack also performed another group of experiments in which he mixed the venom of the cobra with fresh cobra blood and injected the mixture into rats, or injected the serum daily. This was followed by a challenging injection of venom. In conclusion he wrote, "This treatment, therefore, holds out no hope for success as it does not even prolong life." Among other things, he demonstrated the importance of dilution as a factor in determining the lethal effect of cobra venom.

By 1894 there were two groups pursuing studies on venoms and antivenins in France. Phisalix and Bertrand, studying the effect of Vipera berus venom on hedgehogs, found them less susceptible to the venom than guinea pigs (55-56), but it must be admitted, as is similarly overlooked in similar experiments today, that merely multiplying the dose of venom on the basis of the body weight of different kinds of animals is not a justifiable accounting for the argument of immunity. It would be interesting to know what Calmette implied in his statement that "the power of

resistance [in one animal as opposed to another] is therefore beyond doubt." I have commented elsewhere on the significance of the variables that need to be considered in differentiating resistance in different animals as modified by bioavailability, passage across membranes, site of action, metabolism and excretion, and as opposed to immunity (57), but such terms as resistance and immunity are still likely to be confused in the literature.

Phisalix and Bertrand demonstrated that guinea pigs or rabbits inoculated with increasing doses of Vipera berus venom developed an immunity (55,56). Calmette also carried out an extensive number of studies on immunization programs and techniques. He came to one conclusion, that animals immunized with cobra venom "are perfectly immune to doses of viper venom or that of other snakes . . . the serum of the vaccinated animals contains antitoxic substances capable of transmitting the immunity to other animals" (57). This concept of a common antitoxin produced by immunization with one venom and viable for the treatment of all snakebites was commonly held by most investigators of the day.

In 1895, Fraser, viewing the possibility that since serpents were immune to their own venom (a rather projected hypothesis), carried out an experiment in which a cat received one-fifth of a minimum subcutaneous lethal dose of a venom orally at two- to five-day intervals on eight occasions, and then increasing doses until the 116th day when the cat received a dose 80 times larger than the original dose without ill effects. The cat was challenged by one and one-half times the minimum lethal dose and had some local edema and skin necrosis, but survived in good health. Further studies were done with white rats. From these experiments he concluded:

> It would therefore appear that although serpents' venom even in enormous quantities fails to produce any toxic effect

> when introduced into the stomach, it still confers upon the animal a certain and not inconsiderable degree of resistance against the toxic effects of subsequent lethal doses of venom. That it does so by causing an antidotal substance to be present in the blood is also manifest from the result of the kitten which had been fed with milk derived from a parent receiving venom by the stomach (58).

However, Calmette was not able to verify these results (47).

These findings were unknown to the present writer in 1958 when Dr. Barry Campbell of Loma Linda University and I carried out an investigation on mice which had been given venom orally from one-half the intravenous LD_{50} to 100 times that amount over a period of 48 days. They were then challenged with an intravenous LD_{50} of the venom, and although the survival rate was higher in the treated than non-treated animals, the differences were not statistically significant for the numbers of animals studied. This experiment might now be repeated using more sensitive detection techniques such as the ELISA.

In the paper mentioned above, Fraser described his experiments on immunizing rabbits and a horse with gradually increasing doses of venom over several months, or until the animals would withstand 30 to 50 times the minimal lethal dose in the rabbits, or 15 times that dose in the horse. He also experimented with various ways of administering his antivenin for testing procedures: (a) mixing venom and antivenin for 30 minutes and then injecting it "nearly subcutaneously", (b) injecting the venom and then the antivenin into different anatomical sites, (c) injecting the antivenin 30 minutes before the venom, and (d) injecting the venom 30 minutes before the antivenin. The best results were obtained with the mixing technique, and from this he concluded that the experiments "appear to also indicate that the antidotism is rather of the nature of a chemical reaction than of a physiological antagonism" (58).

Fraser also made several statements to the fact that there is a remarkable difference between herbivorous and carnivorous animals, with respect to the resistance to snake venom. He concluded that the effect of serpent venom was probably due to its action on the blood and that antivenin should be injected "in the first instance into the part where the venom had been received, before the ligature had been removed . . . and even before the tissues surrounding the wound had been excised" (58). This is a most thoughtful paper.

Calmette continued his studies on the production of antivenin for preventive and therapeutic applications in snake venom poisoning, first using rabbits and guinea pigs. His basic schedule was to accustom the animal to frequent, repeated, gradually increasing doses of the venom (usually cobra venom). Following Fraser's presentation before the Medico-Chirurgical Society of Edinburgh (59), Calmette furthered his interest in producing an antivenin for clinical use and began to inoculate horses and donkeys. Over a period of 16 months some of his animals became tolerant to 80 times the lethal dose of cobra venom. The antivenin had a neutralizing effect of 20,000 units, that is, "one minimal lethal dose per 1,000 g of rabbits by the dose of 0.1 ml of antivenin expressed in a numerical value of 10,000." That is, 1 ml of the serum could neutralize the minimal lethal dose of venom for 10,000 g of rabbit. Actually, Calmette's antivenin had the ability to neutralize 20,000 g of rabbit. This "antivenomous serum" was then prepared for clinical use. The method he suggested was employed by the Institut Pasteur at Lille, and at laboratories in Bombay, Kasauli, Punjab, Philadelphia, São Paulo, and Sidney (47).

Although the therapeutic value of Calmette's antivenin had been noted by Calmette and Fraser, and both agreed that his cobra antivenin was effective against other venoms, Stephens pointed out that the hemolytic principles of the various venoms were not

identical as far as their affinity to Calmette's antivenin was concerned (60). He concluded that antivenins can act only on the venoms employed and those "allied" to it, but not on all snake venoms. Myers found that cobra venom contained two principles, "cobralysin and cobranervin", the former, a hemolytic substance, could be destroyed by heat, while the latter was unaffected. The cobralysin was neutralized by antivenin while the cobranervin was not (61). It soon became apparent from this and other works that the neutralizing capacity of any one antivenin did not cover all snake venoms.

In the United States, McFarland began a series of experiments in 1899 with the cooperation of the H. K. Mulford Company, employing a modification of Calmette's technique for the production of antivenin. Eventually, he prepared antisera for Crotalus, Agkistrodon and Cerastes. He pointed out the difficulties in producing antibodies against the "irritative" principle of venoms, and demonstrated that Calmette's antivenin was not efficacious for Crotalus venom. He reached the same conclusions as Wolfenden, Phisalix, Bernard, and Calmette with respect to antibodies against nerve toxins. He also noted the many variables that could influence the production of an antivenin. His work was published in five papers between 1900 and 1902 (62).

In 1902, Tidswell prepared an antivenin against the venom of Notechis scutatus, but which showed little neutralizing capacity for other Australian venoms (63). The following year Flexner and Noguchi produced several antivenins against Crotalus venom (64-66). Again, the antivenins had no protective action against non-Crotalus species. Lamb produced an effective immune serum in horses against cobra venom. This and his anti-Vipera russelli antivenin were found to be highly specific (67,68). In that same year Noguchi prepared two antivenins in goats, one for Crotalus adamanteus and the other for Agkistrodon piscivorus. Both had specific neutralizing properties (13).

In 1905, Brazil prepared antivenins against Lachesis (= Bothrops) lanceolatus and Crotalus terrificus (C. durissus terrificus), both being specific (69). In 1907, Ishizaka produced an antivenin against Lachesis (= Trimeresurus) flavoviridis using various modified venom solutions. He observed that rectal administration of the venom led to an appearance of antitoxin in the body of the animal, but that the introduction of the venom per os into the alimentary tract failed to do so (70). Kitashima produced an antivenin against the venom of Lachesis flavoviridis in the goat, ox and horse (71). The reader is referred to the fine compendium of Noguchi (13) for a more detailed accounting of these various earlier works.

It would appear that between 1910 and 1920 there was some slowing down in the progress of our knowledge on snake venom immunology. This is understandable, in view of the war, what this author as a child heard referred to as "The Great War", now commonly termed WWI. Interestingly, in combing the literature for our Bibliography (5) on snake venoms some years ago, I found that several of the younger workers in this field, particularly from France and Germany, had lost their lives during that war and that possibly some of their contributions might have been lost. In any event, because of the limitation on space, I will need to skim over the years since 1910. I have reviewed this period in another book (1), but a far more detailed review will be found in the fine works of Phisalix (72) and Pavlovsky (73).

The "modern period" is reflected by the many contributions of a number of workers, the most prominent of which are two of my colleagues, Paul Boquet of France and Sherman A. Minton of the United States. At the risk of calling their works "historic", I wish to indicate just a few of their contributions I consider to reflect much of our current knowledge and thinking on snake venom immunology.

What has been learned from our history of snake venom immunology? Four intrinsic items come to mind:

1. Responses to venom fractions are highly specific, that is, an antibody can differentiate between various forms of an isomeric antigen.
2. The presence of memory for the first experience provides a basis for subsequent responses to that antigen over a shorter time period.
3. Subsequent responses to the venom antigen are greater, or show amplification in both quality and quantity, although a reaction plateau can be reached after repeated challenges.
4. Failure to maintain self-tolerance may result in autoimmune disease.

Although specificity in itself is a feature of many biological systems such as enzyme-substrate reactions, nucleotide interactions and many facets of embrogenesis, the highly specific recognition process within the immunological system for venoms is distinct, as well as important in its phylogeny and ontogeny. Memory, although common to many biological processes, is so unique to venom immunological responses that it becomes an extremely important factor. The quick recognition of a snake venom antigen or antivenin component, as demonstrated by anaphylaxis, indicates how quickly specificity and memory can be brought into effect. Amplification is not a feature unique to snake venom immunological responses. It is seen in quantitative changes in the liver following the administration of many drugs, and elsewhere. In such cases, however, there is no qualitative change in the reactive material as seen with the antibody response, such as the switch from IgM to IgG production. Thus, although specificity, memory, and amplification may be seen in other biological processes, their relationship in the immunological sytem stands apart. In spite of this uniqueness, there are numerous ways in which the response to the immunological process may be expressed in the patient, and therein lies part of the problem in snake venom poisoning. Poisoning due to the venom of

a snake coupled with the patient's immunological sensitivity to a venom protein can sometimes prove to be a very serious therapeutic problem.

E. Complement

While the serum complement system, at least the healing property of the blood, would appear to have been suspected since antiquity, it was not until the discovery of bacteria and methods for culturing them that studies on the bactericidal activity of blood were initiated. In 1884, Grohmann showed that cell-free plasma was capable of destroying bacteria and other microorganisms (84). Based on the work of Metchnikoff (45), a number of investigators demonstrated that organisms injected into the bloodstream were rapidly cleared by phagocytosis in the spleen and other organs. The bactericidal activity of serum was shown to be destroyed by heating, and a number of workers demonstrated additional effects of various temperatures on the bactericidal property of various sera and other body fluids. This property became known as "alexin" (85) or "cytase" (45).

In an inspiring series of experiments, Bordet distinguished between antibody and complement in immune mechanisms (86), and in 1899 Ehrlich and Morgenroth introduced the word "complement" in describing two combining sites, one for red cells and one for complement (87). Subsequent work by Bordet and Gengou showed that there was only one complement but a great many kinds of antibodies (88). Further work on complement was provided by Buchner (85), Ferrata (84), Brand (90) and Gordon et al. (91).

S. Wier Mitchell and colleagues have been credited by many as the scientists who brought attention to initial concepts about complement and snake venom (50), although Fontana should be credited with carrying out the first definitive studies on the effects of snake venom and the blood (91). Mitchell's various works prior to 1900 certainly indicate his concern for blood

changes associated with those produced by the direct action of the venom. He carried on from the observations of Ewing, who showed that when the serum of rabbits was injected with rattlesnake venom, the bacteriocidal property of the serum was lost (93). Stephens and Myers (94) and Stephens (60) showed that cobra venom, incubated with fresh human or animal blood, produced hemolysis, and that this effect was specifically prevented by the antivenin. They also demonstrated that the hemolysis produced by cobra venom could be inhibited by cobra serum. They further showed that the direct hemolytic activity of cobra serum was destroyed by heating at 68°C for 15 minutes, but that this had no effect of the venom hemolytic factor.

It remained for Flexner and Noguchi to put together some of the ideas of complement as they relate to snake venom, and to carry out a number of experiments that clarified the association of the two (66). Using North American crotalid venoms, they observed agglutination of red cells without lysis when venoms in a 0.5% solution were used, but when the same experiment was done with whole blood both agglutination and lysis occurred. When the temperature was restricted to 0°C only agglutination occurred, and at lower concentrations and higher temperatures lysis took place without agglutination. Agglutination activity was destroyed by heating the venoms to 75-80°C for 30 minutes, but the hemolysis-producing property was stable even at 96-100° for 15 minutes. In these and other experiments, Flexner and Noguchi found "that the active principles of the venom require a second substance to manifest their solvent function upon the blood corpuscles," and that this "masks the opening of a new era of study of the haematoxic actions of venoms." They also demonstrated the difference in haemolysins, which they termed "erythrocytolysins" and "leukocytolysins". Their conclusions were as follows:

1. Venom contains principles which are agglutinating and dissolving for leukocytes.

2. The agglutinating principles may be identical for both white and red cells.
3. The dissolving principles for leukocytes are distinct from those for erythrocytes.
4. In order that solution of venomized corpuscles shall occur, a complement-containing fluid is required.
5. The several varieties of white cells of rabbit blood show different susceptibilities to the action of venom (11,13).

Calmette confirmed the observations of Flexner and Noguchi by demonstrating that venom required an additional substance(s) in the blood serum to produce hemolysis, and that this substance was different from the "serum alexines" in that it did not have activating properties at 62°C (47). Keys (95), under the guidance of Ehrlich, and Keys and Sachs (96) confirmed the work of Flexner and Noguchi, and of Calmette, and explained some of the discrepancies in their findings. They found that there were two kinds of blood corpuscles according to their susceptibility to the hemolytic property of the venom. These were (a) the corpuscles that undergo hemolysis by venom in the absence of a second substance, and (b) the corpuscles that became hemolyzed only when "complements" were present. They also demonstrated that even insusceptible kinds of corpuscles could be dissolved by venom if certain suitably fresh sera were introduced. Further, they showed that susceptible corpuscles contained certain substances capable of activating cobra venom. They labeled these "activators" as endocomplements and found them thermolabile.

Further important contributions to blood changes or venom changes in the presence of blood and/or venoms were provided by Noc (97), von Dungern and Coca (98), Morgenroth and Kaya (99), Sachs and Amorokow (100), Ritz (101), Coca (102), Gordon et al. (91), Pillemer et al. (103), Vogt and Schmidt (104), Alper et al. (105), Alper and Balavitch (106), and Alper et al. (109).

According to Alper, almost all recent studies on complement and snake venom have been done with cobra venom. He points out

that while the mechanisms with crotalid venoms may be different from those precipitated by elapid venoms, the end results are similar. The interested reader will find Alper's review on snakes and the complement system an interesting and important work on the development of this subject (108).

At present, complement is considered as having either "classic" or "alternative" pathways. The classic complement system consists of nine numbered protein components, with the first component being divided into three subunits. The components are thus labeled Cl_q, Cl_r, Cl_s, C2-C9. In reacting with each other sequentially, complement forms products having potent biological effects, including immune adherence, phagocytosis, and cell lysis. The molecular interaction between the first component and IgG or IgM initiates cascading of the classic complement sequence. The alternative or properdin pathway can be activated in the absence of complement binding IgM and IgG by several naturally occurring particulate polysaccharides and lipopolysaccharides, including bacterial endotoxins and IgA aggregates.

The complement system is regulated by inactivators, which are usually enzymes that destroy the primary amino acid sequence of the system, and inhibitors, which do not alter the amino acid chain but rather combine with complement components in such a way as to prevent their further reaction with other components of the system, thus disrupting the cascade.

As Alper has amply put it with respect to snake venoms, "the wheel has now turned full circle with a redirection of scientific interest to the interaction of snake venom and complement after more than half a century of relative dormancy" (108). A renewed interest in cobra venom factor may lead to the structure and precise function of C3, which in turn may yield data on the role of the complement system in allograft rejection and other basic and important biomedical phenomena.

F. Snake Venom Detection

Perhaps one of the earliest techniques for detecting snake venoms in blood or tissues was that proposed by Lamb in 1902. He described a precipitation test for differentiating between "proteids" of different reptile venoms (109). Many years later a modification of the test was employed to detect cobra venom in a fatally envenomated patient (110). In 1957, Minton employed the agar double diffusion method of Ouchterlony to demonstrate the composition of rattlesnake venom. He found that these venoms contained at least four to seven antigenic fractions, three of which appeared commonly shared (79).

In 1967, Russell (111), and also Trethewie and Rawlinson (112), experimented with a simple gel diffusion technique in attempting to detect venom antibodies or antigens. The methods, however, proved to be relatively insensitive and too time-consuming to be of clinical value. The following year, Boche and Russell reported on the use of a passive hemagglutinin test using sheep red blood cells for detecting snake venom in body tissues (113). The test showed accuracy at dilutions greater than 1:200,000 but it was very difficult to perform, as well as being time-consuming. Nevertheless, it was used successfully at the Los Angeles County/University of Southern California Medical Center in a selected group of 25 patients studied over a seven-year period (114). Theakston had difficulty with the method because of controlling conditions for the coupling agents and reagents (115). Indeed, all reagents must be prepared the day of the test, as was done at our Medical Center, if definitive and reproducible results are to be expected.

In 1970 Trethewie again used the Ouchterlony method of gel diffusion in a study on guinea pigs injected with Australian snake venoms. He found considerable overlapping in antigenicity when he used reconstituted dried venoms, but much sharper demarc-

ation when the snake was allowed to bite the guinea pig (116). Ouchterlony techniques were used in determining two deaths following suicides (117,118). Again, the tests were not sufficiently specific to identify the specific snake involved, but they did indicate the probability of snake venom at the family level. Tiru-Chelvam employed immuno-fluorescence techniques for identifying specific sites of localization of snake venoms in vivo. In his summary, he notes that this "study also provides experimental proof of what has been suspected for many years by clinical groups (see reference 119) that the so-called "haemolytic" venoms do indeed have a "neurotoxic" action . . . (120).

In 1974, Greenwood et al. attempted to identify venom in 101 cases of snakebite in Nigeria, employing immunodiffusion and contra-current immunoelectrophoresis. Venom was detected in the wound aspirations of 27 patients, concentrated urine in 19 patients, and blister fluids in 9 patients. In 11 of 26 patients studied, the identity of the offending snake was known, and in these cases the test was positive for that snake (121). In the same year Coulter et al. demonstrated the presence of snake venom in the blood of two patients using a solid phase competitive radioimmunoassay (RIA). The assay demonstrated concentrations of 15 ng per ml in the blood of animals (122). The following year, Sutherland et al. modified the technique for a clinical study in four patients (123). As Coulter et al. subsequently pointed out, the extent of iodination of individual polypeptides can vary considerably (124). The potential for changes at antigenic sites thus makes this test of questionable clinical value (126). This group also noted their experiences with 70 antibody sandwich RIA's in patients bitten by snakes (125).

In their 1978 article (124), Coulter et al. reported the success of their sandwich radioimmunoassay technique, which gave a reliable assay for detecting tiger snake venom in concentrations of 0.1 - 0.4 ng per ml. The authors, however, did not feel

the assay had great clinical value. This concern was also noted by Theakston in 1983, who cited the high equipment cost, requisite technical skill, length of assay time, and shelf-life of the ^{125}I, all of which limited its use to research purposes (115). The RIA, however, demonstrated that labeled antigen-antibody reactions had important laboratory value.

As far back as 1966, Nakane and Pierce (126), and subsequently Massayeff and Maiolini (127) had demonstrated that enzymes might be suitable substitutes for ^{125}I. It remained for Theakston et al. in 1977 to apply the ELISA to the study of snake venoms (128), and in 1978 Pugh and Theakston demonstrated the usefulness of the ELISA in snake venom poisoning in Nigeria (129). Coulter et al. modified the enzyme immunoassay making it possible to obtain determinations in 30-40 minutes (130). Theakston et al. found that the test provided a method of assaying venom potency (131), while Pearn et al. performed ELISA to measure the amount of venom injected into mice following the strike of an Australian common brown snake (132).

Gopalakrishnakone et al. demonstrated the test's usefulness in a study of crude Crotalus durissus terrificus venom and its crotoxin complex (133). Tzeng and Sheik compared two different ELISA enzyme systems to the RIA using cobrotoxin and anti-cobrotoxin. They found the enzyme systems to be as sensitive as the RIA, although the authors felt that the sensitivity with the enzymes was less than expected (134). Since cobrotoxin has only three antigenic determinants, one of which is bound with the solid phase, perhaps with a crude venom the sensitivity would have been greater if there would have been more determinants to bind with the enzyme conjugate.

In 1982 Lwin and Hyint found that the ELISA might be a useful tool for assessing the amount of antivenin to be injected by measuring the amount of uncomplexed venom remaining in the blood. This was determined using optical density. They also found that

the pre-coated plates could be kept at least two weeks without loss of their sensitivity (135).

Chandler and Hurrell cited the need for a rapid field test for medical and veterinary use, particularly in developing countries. They noted that the use of horseradish peroxidase system in field situations was limited by its instability and predilection to inactivation by preservatives. They suggested a urease-based system that employed urea as the substrate, liberated ammonia in the presence of a pH indicator, and provided a bright color change that could be easily read. The system could be stabilized with the preservative sodium azide. They devised a field kit capable of testing for the venoms of five Australian snakes. The system was sufficient to detect venom with a sensitivity of 15 ng/ml in 40 minutes. These results compared favorably to their RIA control sensitivity of 10 ng/ml. The kits were successfully field tested, showing a perfect record of identification for the offending snake in 30 minutes of test time (136).

Theakston described the two principal variants in ELISA systems. The double-sandwich, or direct method, is done by coating a 96-well polystyrene plate with antivenin, washing, adding venom, washing, and then adding a specific antibody. The antivenin is conjugated with an enzyme horseradish peroxidase or alkaline phosphatase, the substrate is added, and the hydrolysis observed through a color change. The second variation involves the indirect method in which plates are coated with venom, antivenin is added, and species-specific conjugated antibody applied to label the antigen-antibody complexes. With both techniques it is possible to observe a significant color change (115). The former method would appear to have a greater potential for clinical use.

The first major study on ELISA and North American snake venoms was carried out by Minton et al. in 1984. In this study they employed mice or rats which had been either injected with a

crotalid venom or bitten by the snake. Serosanguineous fluid from the bite site, affected muscle, heart blood and urine were obtained following the death of the animal. The ELISA employed horseradish peroxidase conjugated to antibody (137). The authors found that there were extensive cross reactions with the venoms of some crotalid species so that positive identification of the species, or genus, could not always be made. Venom could be detected in the muscle of mice receiving 0.5 - 1.5 mg of venom, but rarely in their blood or urine. They found that in most cases Crotalus venom could be distinguished from Agkistrodon venom.

The first clinical use of the ELISA in diagnosis of a clinical case in the United States was described by Banner et al. in 1984 (138). In that patient, initial diagnosis was anaphylaxis due to a bee sting. Subsequently, this diagnosis had to be questioned, and the ELISA performed in blood tissue samples, pleural and pericardial fluids, bite site and brain tissues showed that the offending animal was a rattlesnake. The highest ELISA titers were found in brain tissues. Reactions were strongest for Crotalus scutulatus, then Crotalus atrox. In the other body tissues and fluids the strongest reactions were difficult to determine, with perhaps C. scutulatus giving a stronger reaction than C. atrox. There were fewer cross reactions with the four other crotalids tested. Since only C. scutulatus and C. atrox are found in the roadside area where the child was bitten, and the clinical syndrome would seem more typical of a Mojave rattlesnake bite than of the western diamondback, the probable culprit was C. scutulatus.

In his Master's thesis (139), Hitt employed the ELISA to detect and characterize rattlesnake venoms at the species level in samples from rabbit and human sera. Antibodies were prepared separately in rabbits against the venoms of 11 crotalids and an elapid. Sera were taken at different intervals post-immuniza-

tion. In some studies, the prepared rabbit sera were used, while in others an affinity column product was employed (140). Four antibody and three venom dilutions were studied. There was a consistently high correlation in the results for both the antivenin and the venom dilutions when species-specific products were used. This was less obvious in the cross-reaction studies. In these latter studies, C. s. scutulatus antivenin appeared to give the greatest cross reactions, and Agkistrodon piscivorus the least of the six venoms studied. It is interesting to note the close correlation of these findings with the data presented by Gingrich and Hohenadel (141) on the neutralization of the lethal property of the venom.

Hitt found that the horseradish peroxidase system appeared to be the most practical clinically, but felt that if some of the problems he experienced with the urease system could be remedied, this technique might prove more accurate. Subsequently, Hitt and Russell were unable to detect significant differences between the venoms of the subspecies C. viridis helleri and C. viridis oreganus, nor between A. contortrix contortrix and A. contortrix laticinctus, using the horseradish peroxidase ELISA in 40 venom samples (142). These various studies would seem to indicate that, at least with the North American crotalid venoms, one should be able to distinguish between specific species, provided species-specific venoms and antivenins are compared for the snake in question and then compared with those of related snakes. The differences, however, would usually be quantitative rather than qualitative.

This brings up the question of the value of the test in clinical medicine. In those areas of the world where several genera or even families of venomous snakes abound, such as in Africa, Southeast Asia and Australia, there is little question as to the clinical importance of the ELISA. In the United States, however, it is evident that the quantitative differences

at the species level are difficult to evaluate unless species-specific antibodies are used; and as there are fewer than 20 species of rattlesnakes (Crotalus and Sistrurus), one species each of the water moccasin and copperhead (Agkistrodon), and only two genera of coral snakes (Micrurus and Micruroides), and the clinical syndromes are sufficiently different that few physicians became confused, the test would seem of minor clinical value. Also, in this writer's experience, at least 80% of the victims in the United States see the offending snake and generally can identify it. Furthermore, except in certain areas of the country, overlapping does not present a serious differential diagnostic problem. Lastly, since there is only one commercial antivenin for crotalids in the United States, and since this appears to be effective against the venoms of all North American species, a positive identification at the species level is not imperative for good therapeutic care, even though it would be supportive.

In an unpublished paper presented at a recent symposium, Minton reported that in clinical cases, wound aspirates, serum and urine are the most suitable materials for venom detection (143). He appears to have reached a conclusion similar to that presented by Hitt. Minton's ELISA's were done on human tissue samples sent to him by various physicians throughout the United States. He concludes:

> Current ELISA systems involving snake venoms have rather low specificity. Host cannot readily differentiate venoms of related snakes. Venom antibody detection assays are less satisfactory than those for venom. Non-specific reactions and cross-reactivity are unacceptably high (143).

Perhaps this is reflected by the fact that while both Minton and Hitt can be considered as experts on ELISA techniques, in several of the samples that this writer sent to both, different results were obtained. One might attribute this to changes that could have occurred during shipment of the samples, but this fac-

tor has not yet been determined. Suffice to say, this writer agrees with Hitt and Minton that, with respect to crotalids, the ELISA for crotalid venoms in the United States is of limited value as a clinical tool as the assay now stands.

Historically, the ELISA and CELIA have gradually replaced other methods for detecting and quantitating substances with toxinological properties. The high degree of sensitivity of these assays is due to their properties of specificity, high affinity, reversibility of the binding of antigen to antibody, and to the existence of methods for attaching sensitive, detectable labels (isotopes, free radicals, bacteriophages, etc.) to the antigen or antibody.

The principal objective in preparing an enzyme-labeled antigen or antibody is to obtain a stable conjugate with a high titer of immunoreactivity and enzyme activity. The sensitivity of the assay depends on the binding constant of the antibody and on the specific activity of the labeled immunoreactant. It is well known, however, that the linkage of an enzyme to an antigen or antibody may affect the specificity of an assay if the chemical modification alters or masks key immunological determinants. This may account for some of the discrepancies found in the literature with respect to snake venom ELISA studies.

While the ELISA has a number of advantages over the RIA, the complications of quantitating a label as large as an enzyme in the complex, as compared to an atom of iodine, have not been thoroughly studied immunochemically.

G. Antigenic Relationships

It is well known that there are certain antigenic substances in snake venom that appear to be common to several if not many of these toxins. Based on the work of Bordet and subsequently Nuttall and others, a precipitating substance was first observed by Lamb in 1902 (109). He noted a precipitation cross-reaction

with Vipera russelli venom when using a Naja naja antiserum. Two years later he studied several more venoms and found weak cross-reactions between cobra antivenin and the venoms of the sea snake (Enhydrina schistosa) and the green tree viper Trimeresurus gramineus) (67). He pointed out that the neutralizing property of the antivenin bore no relationship to the precipitation content of the serum, a fact that still has applicability.

Hunter confirmed the presence of common antigens in cobra and Russell's viper serum and venom (144). Flexner and Noguchi (66) also demonstrated that "there is no relation between the degree of protection afforded by and the amount of precipitation present in immune serum." They demonstrated a lack of protection between a Crotalus antiserum and the venoms of the cobra and Russell's viper.

Githins and Butz found that the antiserum for Crotalus atrox neutralized the venoms of five other North American rattlesnake venoms (145). It was less effective against Agkistrodon venoms, and of little value against two South American pit vipers. Subsequently, Githins and Wolff, on the basis of these and other studies, divided North American crotalids into three antigenic groups. The first group contained those snakes producing delayed neurological manifestations, the second, all other Crotalus species, and the third, the Agkistrodon (146). Picado stated that there was a "neurotoxin" in the venom of the Costa Rican arboreal vipers (Bothrops) that is not antigenically homologous with Crotalus durissus "neurotoxin" but which is neutralized by the Bothrops antisera (147). Some years later, Akatsuka demonstrated that antivenin against the habu gave weak reactions with the venoms of a cobra and a pit viper (148).

Kellaway demonstrated that animals immunized against tiger snake venom were not protected against the venoms of the Australian brown snake, black snake, or death adder, although they were resistant to the venoms of the Australian copperhead

and taipan (149). Taylor and Mallick found that antivenin prepared against the cobra and Russell's viper reduced the hemorrhagic activity of the saw-scaled and European vipers (150). Ahuja found that puff adder antivenin neutralized the hemorrhagic activity of Russell's, European, and saw-scaled viper venoms (151). Grasset and Schaafsma found that the venom of the poisonous colubrid Dispholidus typus could not be neutralized by any of five elapid antivenins, but antivenins for an African viper and a South American pit viper gave slight protection (152). A more complete review of recent works on antigenic relationships will be found in the various works of Minton, publications of the proceedings of the International Society on Toxinology, and various issues of Toxicon.

It has also been known since the fine study of Lamb that some venom antigens are common to both a snake's blood and its venom. In some cases, there are stronger reactions between antigens and the snake's serum than between the venom and the antiserum to that venom. Some of these antigens have even been found in the blood of nonvenomous snakes, particularly Natrix and Elaphe. The anticomplement protein in Naja naja venom has been reported to be an altered form of C3 of cobra venom (108).

There is no doubt that there is a definite relationship between morphology and taxonomy on one hand and common antigens on the other. The Elapidae and the Viperidae share some common antigens. The Viperidae and the Crotalidae share many, while the Elapidae and the Crotalidae share few.

The venom of one of the so-called more primitive vipers gives precipitation lines with a number of elapid and viperid antisera, as well as with the venomous colubrid Dispholidus. There is also a very weak cross-reaction between Dispholidus venom and a number of pit viper venoms, as well as that of at least one elapid, Pseudechis papuanus. The carpet viper, Echis carinatus, has several antigens in common with the cobra Naja

nigricollis, and one of these is shared with other species of Naja, Hemachatus and Walterinnesia. Naja haje and N. nigricollis antivenins neutralize moderate amounts of Echis and Cerastes venoms (82).

When one comes to study antigenic relationships at the species and subspecies levels, there appear to be many similarities and fewer differences than at the genus level. In fact, the antigenic differences at the subspecies level may not be evident even with our present-day technology, if such differences exist at all. In comparing the venoms of C. viridis helleri and C. v. oreganus over the 200 miles in which they border or coexist, we could find no antigenic differences in the venoms taken from ten adult specimens of each subspecies of snake captured during July and held for one month prior to milking. Minor individual differences could be demonstrated on recirculating isoelectric focusing, but these were not specific for either subspecies. An antivenin prepared against the former gave equal protection in mice against the latter (153). Minton, however, has obtained distinctly different immunoelectrophoretic patterns with two morphologically similar horned vipers, Pseudocerastes p. persicus and P. p. fieldi (82).

These similarities and differences bring up the question as to the definitiveness of morphological characteristics as the sole determinant on which taxonomy can be based. Antigenic relationships, serum and venom proteins, and the use of the ELISA or similar tests may eventually provide us with a more consistent tool for establishing speciation.

Another problem arises when applying immunodiffusion or immunoelectrophoretic data to the clinical problem. While these tests give us information on antigenic similarities and differences, it must be remembered that they do not necessarily identify those venom components responsible for the deleterious action of the venom, if single components are indeed implicated.

A further question that sometimes arises in relation to the significance of reptile venom antigenic relationships was observed during the preparation of antivenin in goats (154). It was found that in order to produce the exact hemorrhagic effect as that evoked by the crude venom, at least three venom fractions identified by antigen-antibody reactions needed to be employed: one that alters the intimal lining of the capillary wall, the second affects the integrity of the red blood cell membrane, and a third which accentuates these alterations. Indeed, these three components gave about 90% of the crude venom's hemorrhagic activity.

Interestingly enough, one can follow this mechanism of action in the clinical case (155). Early on in most patients it becomes obvious from the swelling and edema following rattlesnake envenomation that excessive amounts of fluid are accumulating in the subcutaneous tissues. It is also obvious that this fluid, for the most part, is coming from the blood. This finding is reflected by the early hemoconcentration found in most patients with moderate to severe Crotalus venom poisoning. One may also find an increase in electrolytes in the fluids of the involved tissues, and this can again be seen by a fall in serum electrolytes. In the more serious poisonings, these phenomena are often followed by the frank loss of blood into the affected tissues and by a fall in hemoglobin and hematocrit values. It is possible that in another venom, even one closely related, that one or two of these components might be recognized by ELISA or other assay techniques, but the third might be missing even though, unless closely quantitated, the reaction might be present.

III. GENERAL PRINCIPLES OF ANTIVENIN PRODUCTION

Most present-day antivenins are refined concentrates of equine serum globulins prepared in a liquid or dried form. These are obtained from horses that have been immunized against a

venom, or a number of venoms. Antivenins have now been prepared for use in the treatment of most types of snake venom poisoning. Their effectiveness in neutralizing both the deleterious and seemingly non-deleterious effects of a specific venom may vary considerably, and depends upon a number of factors. The most important of these factors are the specificity of the antivenin, the titer of antibodies, and the degree of concentration or purification of the final product.

In general, the more specific an antivenin the greater the likelihood that it will neutralize the challenging venom. Some fractions of one venom, however, may be common to a number of venoms from a given genus of snakes, and indeed even from that particular family of snakes, and thus an antivenin prepared from the venom of a single species may protect against the venoms of other snakes. Monovalent antivenins are usually the preparation of choice when the offending snake is known, and a monovalent antivenin is available. The various works of Boquet (74-78) and Minton (79-83) indicate some of the important relationships for monovalent and polyvalent antisera.

Polyvalent antivenins have the advantage of not only mitigating the effects of those venoms used in the immunizing mixture but, as in the case of the monovalent antisera, a number of other related venoms. Antivenin [Crotalidae] Polyvalent (Wyeth), an antitoxin prepared with the venoms of four crotalids (Crotalus atrox, C. adamanteus, C. durissus terrificus and Bothrops atrox), neutralizes the venoms for 17 species of the family Crotalidae, including the toxins of some Agkistrodon (156). In clinical practice, this antivenin is used in the treatment of poisoning by some 65 Crotalidae in North, South, and Central America, and has been employed in the treatment of bites by certain Asian species of Trimeresurus and Agkistrodon. This should not be interpreted to imply that this antivenin is equally effective in all poisonings by Crotalidae. There is considerable variation in the

neutralization titer of this antivenin for some crotalid venoms, as there is for any polyvalent antivenin.

When the species of the offending snake is not known or is in doubt, polyvalent antivenins play a particularly important role. Another advantage of polyvalent sera is that in many areas of the world where many species or even genera of snakes abound, the disposition and packing of a single polyvalent antivenin is much easier than carrying several or many different kinds of antivenins, and since it is often necessary to carry or store more than a single vial of each antivenin, this presents a physical as well as a financial hardship.

The venoms used in the immunizing mixture are generally obtained from a reliable source, or more often provided by the immunizing laboratory itself. In earlier days, the suppliers of these venoms were not always careful about the control of their product, nor were the samples taken from a broad enough spectrum of snakes to be representative of the species. Further, there was a tendency to use only adult snakes, which of course provide more venom than juvenile or small snakes, and with less chance of mishap during the milking procedure.

It has been demonstrated that the venom of snakes of the same species from different geographical locations may possess both quantitative and qualitative differences, and that the venom of juvenile snakes may be different from that of adult snakes of the same species. Thus, it becomes obvious that the immunizing mixture may not always represent the venom possessed by the offending snake.

This problem was addressed by the Food and Drug Administration of the United States in the early 1960's and by the WHO in 1967, but even today ideal or representative mixtures of immunizing venoms have not received the attention they deserve. It might be said that the efficacy of an antivenin more often reflects the nature of the venom sample than the nature of the

snake venom poisoning, as it exists clinically.

Another shortcoming has involved the testing or assaying of antivenins. Up until only a few years ago the only test employed to determine the efficacy of an antivenin was neutralization of the LD_{50}. Today, some producers are employing and evaluating tests for other neutralizing activities, including hemolysis, and the neurotoxic, myotoxic, and other properties. These procedures should result in a more comprehensive understanding of the properties of antivenins, and hopefully better antivenins. In spite of the shortcomings, however, most antivenins are effective against the venom(s) for which they are produced (157). Improved techniques such as the RIA and ELISA should lead the way to the production of much improved antisera during the next decade.

Another problem that has troubled both laboratory and clinical toxinologists has involved the technique for assaying the antivenin ED_{50}. Based on the fine work of Fraser in 1895, the common method for determining the efficacy of an antivenin is to mix a given amount of the material with a given amount of venom, let the mixture stand for 30 minutes, and then inject it into mice, thus determining the neutralizing capacity of the antisera against the LD_{50}, LD_{99}, or some other lethal parameters of the venom. Obviously this has no clinical relationship, but while it lacks that association, the technique is the most widely employed and accepted method of testing antivenins at the present time. This writer has found, as did Fraser and others, that the length of time the two solutions are incubated is a very important factor in the neutralizing capacity of the antivenin. In fact, it may be the most important parameter that is commonly overlooked by the experimenter. In determining the effective titer of an antivenin using this technique, it is imperative that each mouse test reflect doses of <u>exactly</u> the same amounts and volumes, and <u>exactly</u> the same durations of incubation.

One last general consideration relates to attempts to "beef up" antivenins. At the WHO meeting in 1967, A. de Vries and F. E. Russell reported on their attempts to prepare an improved antivenin by adding certain chemically isolated deleterious fractions of venoms to the crude immunizing mixture. These antivenins were termed "hyperimmunized antivenins", and while they showed promise experimentally, only in Israel were they produced commercially.

These studies might be viewed as the forerunners of present monoclonal or polyclonal techniques now being employed in the experimental production of antivenins. Whether or not the raising of polyclonal antibodies for venom fractions will prove to be more effective than previous attempts with hyperimmunized antivenins has yet to be demonstrated. It is certainly hoped that it will be.

Antivenins employed in clinical medicine have been prepared in horses, donkeys, cattle, goats, and rabbits and other kinds of animals for laboratory or experimental use (1). Horses are by far the most frequently employed host, not only because of their size (an average 2,000-pound horse has 60 liters of blood), but because of their longevity, ease of care, competitive cost, high yield of antibodies, and finally their seeming ability to withstand greater insult from the injections of snake venom. In our production of antivenin in goats during the 1970's, we found that the overall cost of producing 100 grams of antivenin in goats was more than twice that for horses.

Most horses used in an immunization program are healthy animals of 1,500 to 2,000 pounds which have been observed and acclimatized for several weeks, and demonstrated to be free of equine diseases, particularly brucellosis and glanders; usually, they have been treated for equine diseases particular to that area prior to immunization.

The venom to be used in the immunizing program is obtained from healthy snakes and generally from the larger individuals of

the species. It would be hoped that the venom would also be collected at different times of the year and from snakes representing the entire distribution area, but as previously noted, this is rarely done. The venom is then desiccated, dried, or lyophilized. In some cases this is done immediately following milking of the snakes (which is preferred), while in some facilities the venom is stored in the refrigerator or frozen and lyophilized when a certain amount is attained. Recent studies indicate that some enzymes may be denatured while the venom is in liquid form even though its lethal property may not be affected.

The venom is then added to a common pool which may vary from 100 grams to several kilograms, from which samples are routinely taken for control studies, and from which venom is taken for the inoculation of the horse. The venom is subjected to some form of sterilization for contaminating bacteria. In most cases this involves incubation with 0.5% formalin for 24-30 hours at 37°C. Irradiation or other methods of sterilization are sometimes used.

In preparing a monovalent antivenin, the neutralizing capacity of the antiserum is checked against a specific venom. In preparing a polyvalent antivenin, several or many venoms are pooled and injected, and following test runs the neutralizing capacity of the resulting antisera are studied against the venoms in the immunizing mixture as well as against other related medically important venoms. Sometimes the battery of testing includes the venoms of snakes seemingly unrelated to the group under study. In the case of the Wyeth antivenin in the United States, during the formative years of testing (1947-1954) a number of investigations involving various combinations of venoms were made. According to Criley (156) at least five different combinations of venoms were studied for their neutralization capacity of the venoms of 11 American snakes. He observed a

strong relationship between the venoms of Crotalus and Bothrops, and subsequently noted the importance of using those venoms having increased proteolytic and neurotropic properties (156). After extensive testing between 1952-1954, he suggested that the polyvalent antivenin for American Crotalidae should be prepared from four venoms: Crotalus atrox, C. adamanteus, C. durissus terrificus, and Agkistrodon piscivorus.

The venom(s) is put into a saline or saline phosphate buffer solution to make a concentration of 1-2%, then mixed with an adjuvant to retard absorption. The kind and amount of adjuvant varies with the producer's experience. The South African Institute of Medical Research, which has had long experience in antivenin production, employs bentonite as an adjuvant (157). Others use Freund's adjuvant, while Wyeth Laboratories uses aluminum hydroxide gel in a 10% concentration. The formula for the mixture of the venoms to be used in the inoculations varies considerably with each producer. In Wyeth's present program, the venoms are mixed in the following proportions:

Crotalus adamanteus	25%
Crotalus atrox	25%
Crotalus d. terrificus	33%
Bothrops atrox	17%

The venoms are then sterilized, usually by incubating the mixture at 37° with 0.5% concentration of formalin for 24 to 30 hours. The current hyperimmunizing schedule at Wyeth Laboratories is shown in Table I. The adjuvant is 10% Amphojel.

In reviewing eight immunizing programs from different laboratories, it would appear that most facilities inject measured amounts of the immunizing mixture into horses over a period of 10-20 weeks. This is usually followed by a rest period of 3-5 weeks, and then a maintenance schedule is introduced in which the horse is given approximately one-third the immunizing dose, and one week later twice that, and one week after that

TABLE 1
Current Hyperimmunizing Schedule

Weeks	Dose (mg)	Saline 0.85% (ml)	AMPHOJEL Vol (ml)	Dose (ml)	Gram % Venom	Injection Route	Sites
1	3	2.7	0.3	3	0.1	subcutaneous	1
2	6	5.4	0.6	6	0.1	subcutaneous	1
3	10	9.0	1.0	10	0.1	subcutaneous	1
4	24	22.6	2.4	24	0.1	subcutaneous	1
5	50	45.0	5.0	50	0.1	subcutaneous	1
6	100	90.0	10.0	100	0.1	subcutaneous	2
7	200	18.0	2.0	20	0.1	subcutaneous	4 (1 for each venom)
8	400	36.0	4.0	40	1.0	subcutaneous	4 " " " "
9	600	54.0	6.0	60	1.0	subcutaneous	4 " " " "
10	750	67.5	7.5	75	1.0	subcutaneous	4 " " " "
11	1000	90.0	10.0	100	1.0	subcutaneous	4 " " " "
12	Rest 9 days, bleed for serum and test for potency.						
13	Rest 5 days.						
14	800	72.0	8.0	80	1.0	subcutaneous	4 " " " "
15	1200	108.0	12.0	120	1.0	subcutaneous	4 " " " "
16	Rest 9 days, bleed for serum.						
17	Rest 2-3 days, bleed for serum.						
	Rest 1 month.						
	Maintenance Schedule						
22	500	45.0	5.0	50	1.0	subcutaneous	4 (1 for each venom)
23	750	67.5	7.5	75	1.0	subcutaneous	4 " " " "
24	1200	108.0	12.0	120	1.0	subcutaneous	4 " " " "
	Rest 9 days, bleed for serum.						
	Rest 2-3 days, bleed for serum.						
	Rest 1 month and repeat.						

Current hyperimmunizing schedule used by Wyeth Laboratories for the production of Antivenin [Crotalidae] Polyvalent.

(F. J. McCarthy, Personal Communication, 1987)

approximately the full dose. In most cases the venom mixture is given subcutaneously, and generally in two to five different areas. The time of bleeding the horses and the amounts vary considerably with the different producers. Some horses have been bled over 70 times during the 15 years they were used for immunization. In general, three to eight liters of blood are taken from a horse at each bleeding. The red blood cells from each bleeding are injected back into the horse at the next bleeding.

The amounts of venom mixture given with each injection over the 10- to 20-week course varies with the specific venom or venoms being given, with the response of the horse, and with the experience of the producer. In the horses we immunized against Crotalus viridis helleri venom in the late 1960's, we gave 5 mg in the first injection and 1,000 mg in the last injection 15 weeks later. Our maintenance injections were 300 mg, 600 mg, and 1,000 mg after a one-month rest period. We bled the animal at 16 weeks and again at 10-day intervals between the 22nd and 38th weeks.

The blood is collected, the serum purified, and the product processed for packaging. The essentials of these procedures are given by the WHO (158). The ideal of the WHO is that an antivenin "should be an almost pure $F(ab)_2$ plasma fraction. The volume of fill will be 5 ml of a 10% protein solution in a 20-ml vial. The 5 ml will be isotonic and contain 2% glycerine at pH 6.4 to 6.8" (158).

The most commonly employed current method for purification of the immunoglubulins is ammonium sulfate precipitation. This is also the method employed by Wyeth Laboratories for their antivenins. Some laboratories purify their products by pepsin digestion, and a few use both ammonium sulfate precipitation and pepsin digestion (see Antivenin List, Table II). Some countries do not state their purification methods, while two or three apparently continue to use crude horse serum.

The final product is then adjusted to the required standard of potency as laid down by the national authority: made isotonic, a preservative added, filtered, checked for pyrogenicity, and then retested. It is then processed into its sterile final form as a liquid or dried product. In general, each vial must contain an excess of 10% of that considered adequate by the national authority. A vial of Antivenin [Polyvalent] Crotalidae contains sufficient material to neutralize 198 mouse LD_{50}s of C. atrox venom, 1,452 LD_{50}s of C. durissus terrificus venom, 858 LD_{50}s of Bothrops atrox venom.

In most clinical papers the amount of antivenin given by a physician is noted as the number of vials. The amount of antivenin in one vial, however, may differ substantially from that in another, for the amounts are usually, but not always, assayed for their neutralization capacity per lot or batch.

One last comment seems indicated: the shelf-life of the product. There is no question that freeze-dried antivenins under vacuum are the most stable, and the liquid preparations the least stable. Thus, attention must be given to the manufacturer's recommendations concerning the product. In general, antivenins in the liquid form, even when stored properly at 5°C, may need to be discarded after two years if their color has changed or a precipitate has formed. They may, however, retain their potency for many years. I have assayed some liquid antivenins after 4 years, when there was no change in their color or consistency, and found their neutralization capacity about the same as when they were prepared. On the other hand, we have tested a Wyeth Laboratory antivenin for its lethal neutralizing property after 20 years of storage and found it to be unchanged. Another sample, however, kept in a rubber-stoppered bottle and opened five times over a 10-year period showed some decrease in its neutralizing titer, as well as a slight change in its color.

In the United States, the shelf-life of the native antivenins is five years. Properly stored, however, these antivenins

TABLE 2
Antivenins Available for the Treatment of Snake Venom Poisoning

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
North America and Mexico			
Wyeth Laboratories Box 8299 Philadelphia, Pennsylvania U.S.A. 19101	*Crotalus d. terrificus* *Crotalus atrox* *Bothrops atrox* *Crotalus adamanteus*	Antivenin (Crotalidae) Polyvalent	Precipitated with ammonium sulphate, and lyophilized.
	Micrurus f. fulvius	Antivenin (Micrurus fulvius)	
Laboratories "MYN", S.A. Av. Coyoacan 1707 Mexico City 12, D.F., Mexico	*Bothrops atrox asper* *Bothrops nummifer* *Crotalus atrox* *Crotalus nigrescens* *Crotalus d. durissus* *Crotalus d. tzabcan* *Crotalus tigris* *Agkistrodon bilineatus*	Snake Antivenin	Enzyme digested, precipitated with ammonium sulphate, and lyophilized.
Gerencia General de Biologicos y Reactivos M. Escobeda 20, C.P. 11400 Mexico D.F., Mexico	*Bothrops atrox asper* *Crotalus b. basiliscus* *Crotalus d. durissus*	Anti-Crotalus and Anti-Bothrops	

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Central America			
University de Costa Rica Cuidad Universitaria Rodrigo Facio San Jose, Costa Rica	*Lachesis muta* *Crotalus d. durissus*	Polyvalent serum	Precipitated with ammonium sulphate. Freeze-dried or liquid.
	Lachesis muta *Bothrops alternatus* *Bothrops asper* *Bothrops jararacussu* *Bothrops jararaca* *Bothrops moojeni* *Bothrops lateralis* *Bothrops cotiara* *Bothrops nasutus* *Bothrops neuwiedi*	Polyvalent	
	Micrurus nigrocinctus *Micrurus fulvius*	Antielapidico	
Laboratory Veterinarios Casilla 5584 Guayaquil, Ecuador	*Bothrops asper* *Bothrops atrox* *Bothrops xanthogrammus*	Antibothropico Polivalente	Precipitated with ammonium sulphate and supplied in liquid form.

(Continued)

Table 2 Continued.

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
South America			
Instituto Nacional de Salud Ave. Eldorado con Carrera Zona G, Bogota, D.E., Colombia	Bothrops atrox Crotalus d. terrificus	Antiophidico Polivalente	Globulin precipitated with ammonium sulphate.
Laboratorio Behrens Ave. Principal de Chapellin Apartado 62 Caracas, 101 Venezuela	Crotalus d. terrificus	Anticrotalico	Foreign-protein-reduced
	Bothrops atrox Bothrops venezuelae	Antibotropico	
Instituto Nacional de Microbiologia Avdo Velez Sarsfield 563 Buenos Aires, Argentina	Crotalus d. terrificus	Anticrotalus	Purified by enzymatic and differential thermocoagulation techniques.
	Bothrops alternatus Bothrops neuwiedi	Bothrops Bivalente	
	Bothrops alternatus Bothrops jararaca Bothrops jararacussu Bothrops neuwiedi	Tropical Polyvalent	
	Micrurus frontalis Micrurus corallinus	Antimicrurus	

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
South America			
Ejercito Argentino Campo de Mayo Batallon 601 Pcia de Bueno Aires, Argentina		Antibothrops bivalente	
		Antimicrurus	
Centro de Zooligia Applicada Universidad de Cordoba Pcia de Cordoba, Republica Argentina		Antibothrops bivalente	
	Crotalus durissus terrificus	Anticrotalus	
Instituto Butantan Ciaxa Postal 65, São Paulo, Brasil	Crotalus d. terrificus	Anticrotalico	Pepsin-digested, and ammonium sulfate precipitation
	Lachesis muta	Antilaquetico	
	Bothrops jararaca Bothrops moojeni Bothrops cotiara Bothrops alternatus Bothrops jararacussu Bothrops neuwiedi	Antibothropico	

(Continued)

Table 2 Continued.

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
South America			
	Crotalus d. terrificus Bothrops jararaca Bothrops moojeni Bothrops cotiara Bothrops alternatus Bothrops jararacussu Bothrops neuwiedi	Antiophidico Polivalente	
Syntex do Brasia S.A. Rua Maria Candida 1813 São Paulo, Brasil	Crotalus d. terrificus and Bothrops sp.	Polivalente antiophidic serum	Pepsin digestion, and ammonium sulphate precipitationi. Final solution contains 18% protein.
	Bothrops alternatus Bothrops atrox Bothrops jararaca Bothrops jararacussu Bothrops cotiara	Antibothropic serum	
Instituto Vital Brazil S.A. Caixa Postal 28 Niteroi Rio de Janeiro, Brasil	Bothrops sp.	Soro Antibotropico	
	Crotalus d. terrificus	Soro Anticrotalico	
	Bothrops sp. Crotalus sp.	Soro Antiofidico Polivalente	

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
South America			
Institutos Nacionales de Salud Departamento de Animales Venenosos Calle Capac Yupanqui n 1400 Apartade n 451, Lima, Peru	Bothrops atrox Bothrops bilineatus smaragdinus Bothrops castelnaudi Bothrops brazili Bothrops pictus	Suero antibotropico polivalente	Purified and lyophilized
	Lachesis muta	Suero antilachesico	
	Crotalus d. terrificus	Suero anticrotalico	
Europe			
Institut Pasteur Production 3 Boulevard Raymond-Poincaré 92430, Marnes-la-Coquette, France	Vipera ammodytes Vipera aspis Vipera berus	Ipser Europe	Concentrated and purified to 12-13% protein.
	Bitis arietans Bitis gabonica Echis carinatus Hemachatus haemachatus Naja haje Naja melanoleuca Naja nigricollis Naja nivea	Ipser Afrique	

(Continued)

Table 2 Continued.

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Europe			
	Vipera ammodytes Vipera lebetina obtusa Vipera palestinae Cerastes cornutus Cerastes vipera Echis carinatus Naja naja Naja haje	Near and Middle East	
	Dendroaspis angusticeps Dendroaspis jamesoni Dendroaspis polylepis Dendroaspis viridis	Dendroaspis	
Institut Mérieux 17 Rue Bourgelat 69002 Lyon, France	Vipera aspis Vipera berus	Sérum anti-venimeux purifié Mérieux	
Laboratoires Lelong 45200 Amilly, France	Vipera ammodytes Vipera aspis Vipera berus	Sérum anti-venimeux Lelong	

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Europe			
Hoechst Aktiengesellschaft Postfach 80 03 20 D-6320 Frankfurt am Main 80 West Germany	Vipera ammodytes Vipera berus	Europe	Prepared by pepsin digestion, and ammonium sulphate precipitation. Final solution contains 16% protein. Supplied in liquid form.
	Bitis arietans Bitis gabonica Bitis carinatus Naja haje Vipera lebetina	North and West Africa	
	Bitis arietans Bitis gabonica Dendroaspis polylepis Naja haje	Central Africa	
	Echis carinatus Naja haje Vipera ammodytes Vipera lebetina	Near and Middle East	
Twyford Pharmaceutical Services Deutschland GmbH, Postfach 21 08 05 D-6700 Ludwigshafen am Rhein West Germany	Agkistrodon rhodostoma	Malayan pit viper snake antivenin	
	Calloselasma		

(Continued)

Table 2 Continued.

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Europe			
Istituto Siereoterapico e Vaccinogeno Toscano "Sclavo" Via Fiorentine 1, 53100 Siena, Italy	Vipera ammodytes Vipera aspis Vipera berus Vipera ursinii	Antiviperin	Enzyme refined and supplied in liquid form.
Istituto Sieroterapico Via Darwin 20, Milano, Italy	Vipera ammodytes		Enzyme refined and supplied in liquid form.
Institut Sérothérapique et Vaccinal Suisse Case Postale 2707 3001 Berne, Switzerland	Vipera ammodytes Vipera aspis Vipera berus	Sérum Antivenimeux	
Serotherapeutisches Institut Wien Triester Strasse 50 A-1100 Vienna, Austria	Vipera ammodytes Vipera aspis Vipera berus Vipera lebetina	Schlangengiftserum, "Sero"	
Institute of Immunology Rockefellerova 2 Zagreb, Yugoslavia	Vipera ammodytes	Antiviperinum	Digested with and precipitated with ammonium

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Europe			
Chemapol Foreign Trade Company, Ltd. Kodanska 46, 100 Praha 10, Czechoslovakia	Vipera ammodytes	Antivipera ammodytes	Pepsin digested and precipitated with ammonium sulphate. Supplied in liquid form.
Institute of Epidemiology and Microbiology Sofia, Bulgaria	Vipera ammodytes (This institute also prepares the same serum in crude form for Albania.)	Monovalent	Ammonium sulphate precipitation.
Ministry of Public Health 101 431, GSP 4 Moscow K-51, U.S.S.R.	Echis carinatus Naja naja Vipera lebetina	Polyvalent	No confirmation indicating product or processing.
	Naja naja oxiana	Anti-Naja	
	Vipera lebetina	Anti-Vipera	
Africa			
Institut Pasteur Rue de Docteur Lavéran Alger, Algérie	Cerastes cerastes Vipera lebetina	Antivipérin	

(Continued)

Table 2 Continued.

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Africa			
Institut Pasteur Place Charles-Nicolle Casablanca, Morocco	Cerastes cerastes Vipera lebetina	Antivipérin	
Institut Pasteur 13 Place Pasteur Tunis, Tunisia	Cerastes cerastes Vipera lebetina	Antivipérin	
Al Algousa Sharea Alvezara Cairo, Egypt	Cerastes cerastes Cerastes vipera	Anti-Vipera	
	Naja haje Cerastes cerastes Cerastes vipera	Polyvalent	
The South African Institute for Medical Research P.O. Box 1038 Johannesburg 2000 Republic of South Africa	Hemachatus haemachatus Naja nivea Naja haje Naja melanoleuca Naja nigricollis Dendroaspis angusticeps Dendroaspis jamesoni Dendroaspis polylepis Bitis arietans Bitis gabonica	Polyvalent	Digested with pepsin and precipitated with ammonium sulphate.
	Echis carinatus	Echis	

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Africa			
FitzSimmon's Snake Park P.O. Box 1 Snell Parade Durban, South Africa	Dendroaspis angusticeps Dendroaspis jamesoni Dendroaspis polylepis	Dendroaspis	Digested with pepsin, precipitated with ammonium sulphate, and dialyzed.
	Hemachatus haemachatus Naja nivea Bitis arietans Bitis gabonica	Polyvalent	
Middle East			
Ministry of Health Dept. of Laboratories P.O. Box 6115 Jerusalem 91060, Israel	Echis coloratus	Anti-Echis	Whole venom plus resin-bound "neurotoxin" used as antigen. Supplied as globulin fraction of horse serum in liquid form.
	Vipera palaestinae	Anti-Vipera	
Institut d'Etat des Sérums et Vaccins Razi P.O. Box 656 Teheran, Iran	Naja naja oxiana	Anti-Cobra	Prepared by pepsin digestion, and ammonium sulphate precipitation.
	Vipera lebetina	Anti-Lebetina	

(Continued)

Table 2 Continued.

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Middle East			
	Echis carinatus	Anti-Echis	
	Pseudocerastes persicus	Anti-Persica	
	Vipera xanthina	Anti-Latifi	
	Agkistrodon halys	Anti-Agkistrodon	
	Naja naja oxiana *Vipera lebetina* *Echis carinatus* *Pseudocerastes persicus* *Vipera xanthina* *Agkistrodon halys*	Polyvalent	
Asia			
Haffkine Biopharmaceutical Corp., Ltd. Parel, Bombay India	*Bungarus caeruleus* *Naja naja* *Vipera russelli* *Echis carinatus*	Polyvalent	Digested with pepsin, concentrated and lyophilized.
Central Research Institute Kasauli, India	*Naja naja*	Anti-Naja	Enzyme-refined globulin in liquid and lyophilized forms.
	Bungarus caeruleus	Anti-Bungarus	
	Vipera russelli	Anti-Vipera	

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Asia			
	Echis carinatus	Anti-Echis	
	Naja naja *Bungarus caeruleus* *Vipera resselli* *Echis carinatus*	Polyvalent	
National Institute of Health Biological Products Div. Islamabad, Pakistan	*Vipera russelli*	Monovalent Vipera	
	Echis carinatus	Monovalent Echis	
	Naja sp. *Bungarus sp.* *Vipera russelli* *Echis carinatus*	Polyvalent Anti-snake Serum	
Industrie and Pharmaceutical Corporation Rangoon, Burma	*Naja n. kaouthia*	Mono-cobra	Precipitated with ammonium sulphate and lyophilized.
	Vipera russelli siamensis	Mono-Vipera	
	Naja n. kaouthia *Vipera russelli siamensis*	Biovalent	
Queen Saovabha Memorial Institute Rama 4 Road Bangkok, Thailand	*Bungarus fasciatus*	Bungarus	
	Naja naja	Cobra	
	Ophiophagus hannah	King Cobra	

(Continued)

Table 2 Continued.

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Asia			
	Vipera russelli	Russell's Viper	
	Agkistrodon* rhodostoma	Malayan Pit Viper	
	Trimeresurus albolabris Trimeresurus erythrurus *Calloselasma	Bivalent	
Perusahaan Umum Bio Farma (Pasteur Institute) Jl, Pasteur 20 P.O. Box 47 Bandung, Indonesia	Agkistrodon* rhodostoma Bungarus fasciatus Naja sputatrix *Calloselasma	Trivalent anti-venom serum	Purified serum supplied in liquid form.
Shanghai Vaccine and Serum Institute 1262 Yang An Road Shanghai, China	Agkistrodon halys Agkistrodon acutus	Hamushi	Precipitated with ammonium sulphate and lyophilized.
National Institute of Preventive Medicine 161 Kun-Yang Street Nan-Kang, Taipei Taiwan	Agkistrodon acutus Naja naja atra Bungarus multicinctus	Agkistrodon Naja Bungarus	Immunized with formalin--toxoid venom. Ammonium sulphate precipitated, and supplied

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Asia			
	Naja naja atra Bungarus multicinctus	"Polyvalent neurotoxic antvenins"	in liquid or lyophilized form.
	Trimeresurus mucrosquamatus Trimeresurus gramineus	"Polyvalent haemorrhagic antivenin"	
The Chemo-Sero-Therapeutic Research Institute 668 Okubo Shimizu Kumamoto 860, Japan	Trimeresurus flavoviridis	Habu	Pepsin digestion, ammonium sulphate precipitation, and lyophilized.
	Agkistrodon halys	Hamushi	
Takeda Chemical Industries, Ltd. Higashi-Ku Osaka, Japan	Agkistrodon halys	Hamushi	Pepsin digestion, ammonium sulphate precipitation, and lyophilized.
Research Institute for Microbial Diseases Osaka University Kita-ku Osaka, Japan	Agkistrodon halys	Hamushi	Pepsin digestion, ammonium sulphate precipitation, and lyophilized.

(Continued)

Table 2 Continued.

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Asia			
Kitasato Institute Minato-ku Tokyo, Japan	*Agkistrodon halys*	Mamushi	Pepsin digestion, ammonium sulphate precipitation, and lyophilized.
Chiba Serum Institute 2-6-1 Konodai, Ichikawa Chiba, Japan	*Agkistrodon flavoviridis* (absorbed habu toxoid)	Habu	Pepsin digestion, ammonium sulphate precipitation, and lyophilized.
	Agkistrodon halys	Mamushi	
Serum and Vaccine Laboratories Alabang Mutinlupa Rizal, Philippines	*Naja naja philippinensis*	Cobra	Concentrated and purified.
Australia			
Commonwealth Serum Labs 45 Poplar Road Parkville, Victoria 3052 Australia	*Acanthophis antarcticus*	Death adder	Prepared by pepsin digestion, and ammonium sulphate precipitation. The products are dialyzed and ultrafiltered to a final concentration of 17% protein.
	Notechis scutatus	Tiger snake	
	Enhydrina schistosa	Sea snake	
	Oxyuranus scutellatus	Taipan	
	Pseudonaja textilis	Eastern brown snake	
	Pseudechis australis	Brown snake or Mulga	

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Australia			
	Oxyuranus scutellatus *Acanthophis antarcticus* *Notechis scutatus* *Pseudechis australis* *Pseudonaja textilis*	Polyvalent (Australia-New Guinea)	

should show efficacy for 10 or more years, but the legal complications attending their use beyond five years make it necessary to advise that the antivenin be discarded or used for laboratory research purposes. A listing of antivenin producers is shown in Table II.

Acknowledgements: The author wishes to express his appreciation for the advice and assistance of S. A. Minton, W. S. Jeter, N. B. Egen, F. J. McCarthy, N. C. Bucknall, M. R. Russell and C. Morrow. Part of this work was prepared while the author was the George C. Griffith Scholar of the American College of Physicians. Studies noted as unreported, 1984-1987, were supported by NS 17744 to the author and a grant from R. Sutro.

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APPENDIX: GLOSSARY OF TERMS

ACQUIRED IMMUNITY: Immunologic resistance developed after birth as a result of previous exposure. Sometimes referred to as "specific active immunity".

ACTIVE IMMUNIZATION: Induction of a state of immunity, usually to a specific antigen, produced by the individual's or animal's own immune system.

ADJUVANT: A substance that can increase the specific production of an antibody to an antigen by increasing its size or length of survival in the circulation.

AFFINITY: The intrinsic binding power of an antibody-combining site with an antigenic substrate binding site.

AGGLUTINATION: Antigen-antibody reaction in vitro in which contact results in aggregates or clumps.

AGGLUTININ: A multivalent molecule that causes agglutination by direct interaction with its corresponding antigen. IgM is a particularly potent agglutinin.

ALLELE: An inherited variant of a gene.

ALLERGEN: A substance capable of inducing an allergic reaction.

ALLERGIC: A state of altered reactivity, generally denoting hypersensitivity.

ALLERGY: An immune reaction resulting in a reaction to some tissues or cells, usually through a hypersensitivity reaction.

ALLOGENIC: Having a different genetic constitution, usually used to describe intraspecies antigenic differences (xenogeneic).

ALLOSTERIC TRANSFORMATION: The binding of C1 to the antigen-antibody complex, resulting in a shape change that exposes a new site (allosteric site) by which it can react with another complement protein (C4).

ALLOTYPE: A genetic marker at an individual locus, usually inherited as alternatives.

ANAPHYLAXIS: A state of severe hypersensitivity to a foreign substance caused by the release of vasoactive amines, and triggered by the interaction of cell-bound antibody with the antigen.

ANAPHYLOTOXINS: Substances that degranulate mast cells on being released during an anaphylactic reaction. Histamine and serotonin are major anaphylotoxins.

ANERGY: Absence of immunological reactivity.

ANTIBODY: A molecule produced in response to exposure to an antigen and which has the property of combining specifically with that antigen at its antigen-combining site.

ANTIGEN: A substance of relatively high molecular weight that is capable of mitigating the production of an antibody specific to itself.

ANTIVENIN (ANTIVENENE, ANAVENIN, ANTIVENIMEUX, ANTIVENINIUM, ANTIVENOM): An antitoxin prepared by immunizing animals against a specific venom or venoms, processing the sera, and preparing it for use in humans or other animals. Antivenin is preferred to the other terms on the basis of historical precedent and the implication that it identifies a specific technique, that is, the immunization of animals. The term antivenom is frequently employed to denote any substance that has an action against a

venom, such as tannic acid, extracto de guaco, $KMnO_4$, strychnine, musk, or any other substance. Antivenin also enjoys a far more world-wide usage than the other words. For these reasons antivenin seems a more appropriate and less confusing term.

ANTIVENIN INDEX: A listing of the antivenins available in zoos and universities throughout the United States for the treatment of native and exotic snakes.

ATTENUATED: To be rendered less virulent.

AUTOANTIBODY: An antibody directed against self antigens (autoantigens).

AVIDITY: The combining power of an antibody with its antigen; related to the affinity and the valencies of the antibody and its antigen.

BLOCKING ANTIBODY: An "incomplete" antibody capable of coating the red cell determinant to render it partially or completely blocked and inagglutinable by antibodies of the same specificity.

CAPPING: The process of redistribution of cell-surface determinants to one small part of the cell surface.

CARRIER: An immunogenic molecule to which a hapten is coupled in such a way as to induce an immunological response.

CLONE: A family of cells or organisms of identical genetical constituents derived asexually from a single cell by repeated division.

COBRA VENOM FACTOR (CVF): A C3b analogue isolated from cobra venom. It has the property of activating the 'alternative pathway' of complement activating and destroying C3-C9.

COLD AGGLUTININ: An agglutinin whose optimum temperature of reactivity is in the cold, whose potency decreases with increases in temperature, and whose reaction at 37°C is usually negative.

COMPLEMENT: A complex group of 11 distinct glycoproteins found in the blood serum and other body fluids that react with one another sequentially in a cascading reaction to form potent biological effects, including immune adherence, phagocytosis and cell lysis. Complement factors are designated by the letter C: C1, C2, etc. C1 is composed of three subunits: C1q, C1r, and C1s. Activation of the complement system occurs with IgM or IgG. Chemically, these proteins are fairly large molecules, 75,000 - 240,000 daltons.

COMPOUND ANTIGEN: A combination of more than one antigen against which a single antibody appears to be directed.

CYCLIC AMP: An intracellular mediator having a particularly important effect on the activity of microtubules and other contractile elements of a cell.

CYTOPHILIC: Having an affinity for cells. Usually applied to antibodies which bind to macrophages.

DOUBLE DIFFUSION: Immunochemical analysis of antigenic relationships, pioneered by Ouchterlony.

EFFECTOR CELL: A cell actually carrying out a specific function, such as cell-mediated cytotoxicity.

ELISA: An acronym for enzyme-linked immunosorbent assay. This assay utilizes the principle of a solid phase (e.g., beads or microtiter plate wells) coated with antigen or antibody and an indicator reagent, antibody or antigen, respectively, to which an enzyme has been conjugated or "linked".

ENDOTOXIN: Lipopolysaccharides localized in cell walls.

EXOTIC SNAKES: Foreign, or those non-native to the United States.

Fab FRAGMENT: The fragment of the antibody molecule capable of antigen binding.

FREUND'S ADJUVANT: A water-oil emulsion of antigen-killed M. tuberculosis, usually in the oily phase (complete Freund's adjuvant). Incomplete Freund's adjuvant contains no organisms in the oil phase.

HAPTEN: A small molecule which will combine with antibody but which is not capable of evoking an antibody response in itself.

HEMAGGLUTININ: A molecule capable of agglutinating red blood cells.

HETEROLOGOUS: Usually used to denote inter-species antigenic differences.

IDIOTYPE: An antigenic marker for the antibody combining site. The antigen is found in the region of the antibody secreted by a single clone of lymphoid cells. Antibodies of different specificities have different idiotypes.

IgG: The predominant immunoglobulin class present in human serum.

IMMUNE ADHERENCE: A "glue-like" phenomenon occurring when a particular antigen, its homologous antibody, and complement unite.

IMMUNITY: Resistance to extraneous, foreign matter as determined by the immune system.

IMMUNOCONGLUTININS: Antibodies (often autoantibodies) formed to complement components or their breakdown products, often autoantibodies.

IMMUNOFLUORESCENCE: The method involving the use of fluorochrome-labelled antibody to cellular determinants.

IMMUNOGENIC: Producing immunity. Autigenic.

IMMUNOGENICITY: The ability of an antigen to stimulate antibody production.

IMMUNOGLOBULIN: An antibody containing globulins, including those proteins without apparent antibody activity.

IMMUNOLOGIC ENHANCEMENT: The prolongation of the survival of an allograft from the action of a humoral antibody as against donor-histocompatible antigens that are lacking in the host.

INCOMPLETE ANTIBODY: An antibody that sensitizes red cells suspended in saline but fails to agglutinate them.

INHIBITION: The blocking of the normal reaction between an antigen and its corresponding antibody.

ISOANTIBODY: An antibody that reacts with an antigen present in another member of the same species but not in the animal itself.

ISOANTIGEN: An antigen that elicits antibody formation in another member of same species not genetically identical.

MAJOR CROSSMATCH: A compatibility test used to detect the presence of antibody in the recipient's serum: donor's red cells versus recipient's serum.

MINOR CROSSMATCH: A compatibility test used to detect the presence of antibody in the donor's serum: donor's serum versus recipient's red cells.

MONOCLONAL: Derived from a single-cell clone, usually immunoglobulin, to denote unusual homogeneity.

MONOVALENT: A single antigen or antibody. In general, monovalent antivenins are prepared with the venom of a single species of snake, although the antivenin may mitigate the effects of antigens of several or more snakes of the same or closely related genera.

NATURALLY OCCURRING ANTIBODIES: Antibodies that occur without an apparent stimulus. Also known as non-red-cell-immune antibodies or innate antibodies.

PANAGGLUTINATION: The reaction of red cells, irrespective of blood group, with all human sera.

PASSIVE ANTIBODY: An antibody which, when injected into an individual, provides temporary immunity.

PHAGOCYTOSIS: Ingestion of a solid or semisolid material into a cell by closing off an invagination of the protoplasm. The process requires the activity of contractile elements of the cells and aerobic respiration. The contents of the phagosome are usually digested by the discharge of cathepsins and other enzymes into the phagosome.

PLASMA CELLS: A terminally differentiated antibody-forming cell with a short half-life.

POISONOUS ANIMAL: Those creatures whose tissues, either in part or in their entirety, are toxic. Poisoning by these animals usually takes place through ingestion of their flesh. Sometimes called cryptotoxic animals.

POLYAGGLUTINATION: The agglutination of red cells by most human sera, irrespective of blood group.

POLYVALENT: Referring to several or many antigens or antibodies, often of different species, genera or even families.

PRECIPITATION: The reaction of soluble antigens with antibody, resulting in arcs or flocculation of the complexes in a gel medium.

PRECIPITIN: An antibody that reacts with its corresponding antigen to form a precipitate.

PRIMARY RESPONSE: The initial response to a foreign antigen.

PSEUDOAGGLUTINATION: The clumping of cells caused by agents other than antibodies.

PYROGENS: Thermostable, filter-passing substances that may cause febrile reactions when injected into a recipient. Probably of bacterial origin.

REAGENT RED CELLS: Red cells used in the laboratory for testing purposes.

RIA: A variety of immunological methods in which a radioactive isotope is used to detect antigens or antibodies.

SALINE ANTIBODY: An antibody that reacts with saline-suspended red cells.

SENSITIZATION: Stimulation by an antigen that renders a person liable to form antibodies.

SPECIES-SPECIFIC: Antigens or antibodies restricted to a particular species.

SPECIFICITY: The affinity between an antigen and its corresponding antibody.

SUBGROUPS: With respect to antigens or antibodies, subdivisions; often weakened forms.

SYNERGISM: The cooperative action between venom components.

TOLERANCE: A state of specific immunological unresponsiveness induced by exposure to antigen.

TOXIN: A substance derived from the tissues of a plant, animal, or microorganism which has a deleterious effect on another plant or animal. The word is usually used to denote a venom or poison fraction, although it is sometimes used to indicate the whole venom.

TOXOID: Toxins that have been modified to minimize their deleterious effects, while still retaining their immunogenic and antigenic properties.

VACCINATION: The inoculation or ingestion of organisms or antigens to produce immunity to those organisms or antigens in the recipient.

VENOMOUS ANIMAL: An animal having a venom gland or highly specialized group of secretory cells, a venom duct (although this is not a consistent finding), and a structure for delivering the venom, such as a sting, tooth or fang.

WARM ANTIBODY: An antibody that reacts optimally at 37°C.

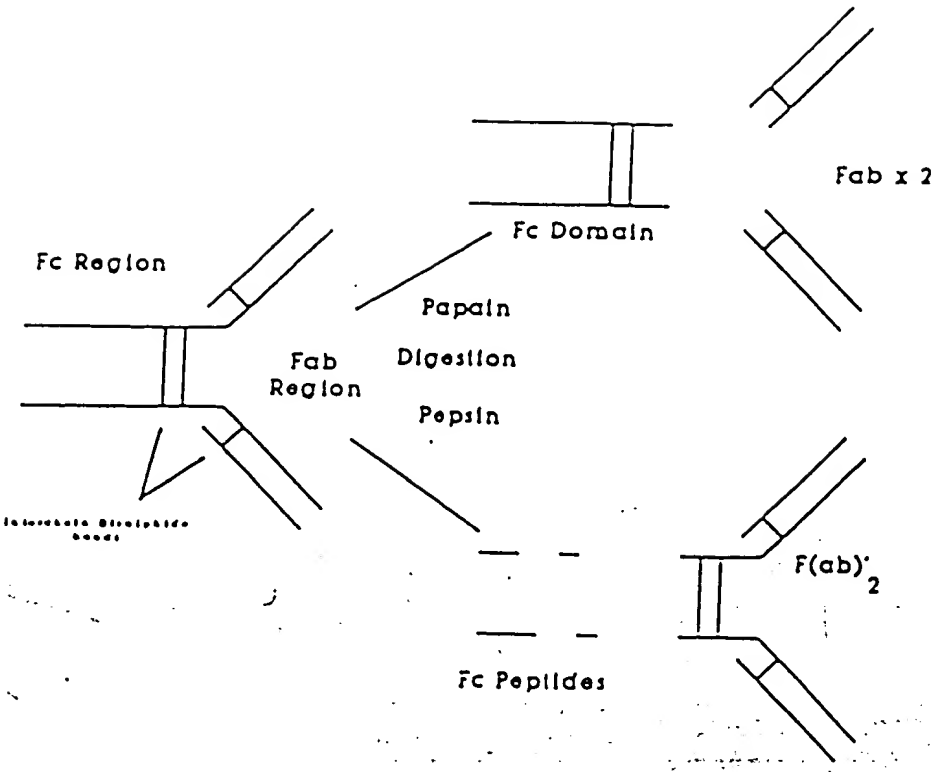
J. TOXICOL.-TOXIN REVIEWS, 7(1), 83-86 (1988)

EDITORIAL

RECOMBINANT TOXINS: WE HAVE LITTLE TO FEAR BUT FEAR ITSELF

Much has been said and written about the enormous potential of recombinant DNA and the new biotechnologies to enrich modern society. Unfortunately, too much has remained potential and too little realized. A concensus has developed that the major problems preventing society from reaping the benefits of recombinant DNA technology are fear of unknown dangers and the regulatory jungle created in response to that fear. Molecular biologists profess dismay at the public's reaction, most notably the public's reluctance to accept without question their assurances that no substantial dangers are involved. The molecular biologists have forgotten (or repressed) the memory that their own first reactions to the dawn of the recombinant DNA era were fear, moratorium, and regulated activity (albeit mostly self-imposed). Furthermore, they are not doing a very effective job of explaining why or how they achieved such a complete change of heart. If the change has not been complete, it has at least been very extensive. Agreement on the innocuousness of recombinant DNA technology is so nearly complete among molecular biologists that even the creationists seem better able to recruit expert opinion to support their cause than do the lawyers who are seeking to contain recombinant DNA within a web of government regulations.

What has caused this change in attitude? Why do the molecular biologists no longer fear the creation of some new and terrible Andromeda strain that would spread a hideous plague upon the world? If pressed, they might say, "Well, maybe if you transfer genes for cobra venom components or some other deadly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Basic Immunology

Immune Mechanisms in Health and Disease

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Elsevier Science Publishing Company, Inc.
52 Vanderbilt Avenue, New York, New York 10017

Sole distributors outside the United States and Canada:
Elsevier Applied Science Publishers Ltd.
Crown House, Linton Road, Barking, Essex IG11 8JU, England

Library of Congress Cataloging-in-Publication Data

Sell, Stewart, 1935-

Basic immunology: immune mechanisms in health and disease.

Includes bibliographies and index.
1. Immunology. I. Title
QR181.S357 1987 616.079 86-32990
ISBN 0-444-01136-2

Current printing (last digit)
10 9 8 7 6 5 4 3 2 1

Manufactured in the United States of America

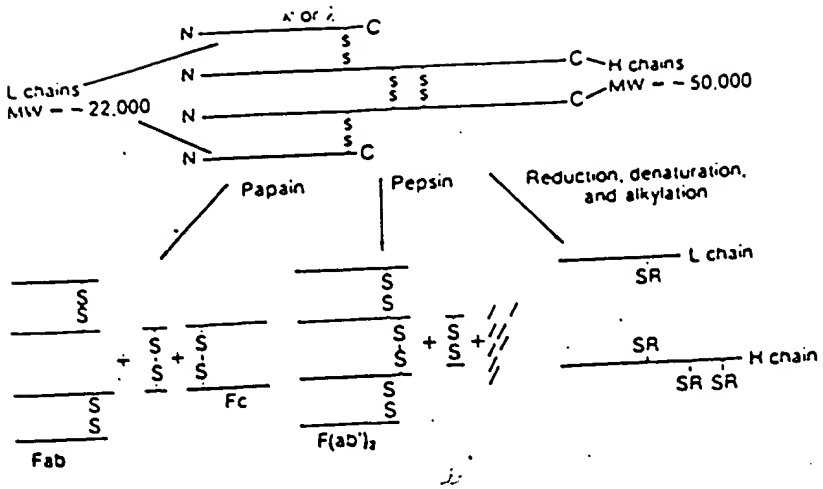
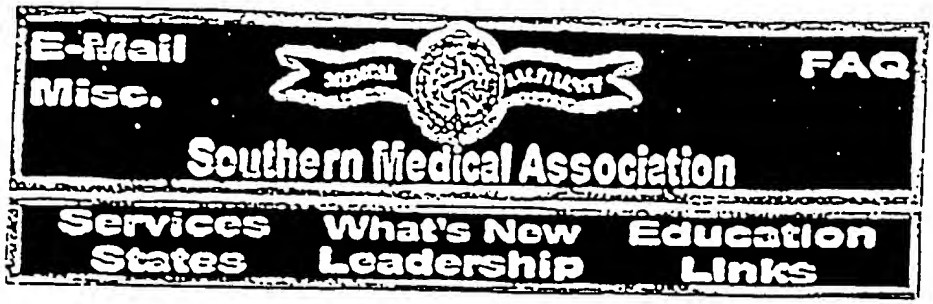


Figure 6-3. Human immunoglobulin fragments. The intact IgG molecule may be fragmented by different reagents into subunits. Digestion with papain occurs on the amino side of the interchain disulfide bond and results in three major fragments, two Fab and one Fc, and a minor fragment. Fab fragments consist of an L-chain and the amino half of an H-chain joined by a disulfide bond. The Fc fragment consists of the carboxy halves of H-chains joined by a disulfide bond. An additional small peptide from the middle of the heavy chains containing a disulfide bond is also produced. The Fab fragment contains an antigen-binding site and reacts with, but does not precipitate, antigen because it is monovalent. The Fc portion is responsible for biological properties such as complement fixation. Digestion with pepsin occurs on the carboxy side of the interchain disulfide bond and results in two F(ab') fragments joined by a disulfide bond because one of the disulfide bonds joining the H-chains is preserved. This fragment, $F(ab')_2$, reacts with and precipitates antigen because it is divalent (contains two antigen-binding sites). Additional peptide fragments, some containing disulfide bonds, are produced by the action of pepsin, presumably due to further digestion of the Fc fragment. Reduction of disulfide bonds, alkylation of free SH groups ($R - CH_2CONH_2$), and denaturation of ionic and hydrogen bonds result in liberation of polypeptide chains — two L-chains (MW 22,000) and two H-chains (MW 50,000). Each polypeptide chain contributes to the antigen-binding site of the intact Fab fragment. That portion of H-chain present in the Fab fragment is called the Fd piece.

maturing fetus and may be the first immunoglobulin class representing a given antibody specificity following immunization (primary response). IgM occurs as five H_2L_2 units joined to each other by disulfide bonds located on the Fc part of the molecule and to the J-chain; its molecular weight is 900,000. IgM is found mainly in the intravascular fluids (80%). It is also the most efficient class of immunoglobulin in fixing complement and therefore is highly active in cytotoxic and cytolytic reactions.

IgM does not normally cross the placenta from mother to fetus, but may be produced actively by the fetus prior to birth, especially if the fetus has been exposed to antigens by infection. Thus IgM antibodies in the cord blood of the fetus are evidence of fetal immunization by exposure to infectious agents.



Levels of Therapeutic Antivenin and Venom in a Human Snakebite Victim

CHARLOTTE L. OWNBY, PhD, SANDRA L. REISBECK, DVM, Stillwater, OK; and RUSSELL ALLEN, MD, Oklahoma City, OK

ABSTRACT: An enzyme-linked immunosorbent assay was used to measure the levels of therapeutic antivenin (Antivenin [Crotalidae] Polyvalent, Wyeth-Ayerst) in serum and the levels of venom in the urine of a patient bitten by a western diamondback rattlesnake (*Crotalus atrox*). Serum and urine samples were taken on admission, during hospitalization, and during follow-up until 5 months after the bite. Photographs were taken of the bite site on admission to the hospital and during follow-up. Serum levels of therapeutic antivenin were highest between 1 and 3 days after the bite but were still detectable 46 days as well as 4 months after the bite. *Crotalus atrox* venom was detectable in the urine 4 days after the bite and was still measurable 6 days after the bite.

Venomous snakebite is a serious medical problem in many parts of the world, and the most widely accepted treatment is serotherapy with either specific antivenin or more commonly with a polyvalent antivenin. In the United States, serotherapy using Antivenin (Crotalidae) Polyvalent (Wyeth-Ayerst, Marietta, Pa) is still the recommended treatment for serious snakebite cases.1-3 Debate continues about the appropriateness of using antivenin, the route of injection, the dose to give, and when to administer it. This is due, in part, to the lack of knowledge about the pharmacokinetics of venom in the human patient. The enzyme-linked immunosorbent assay (ELISA) has been used to measure the levels of venom in the serum of snakebite patients in an effort to determine the pharmacokinetics of venom, to measure the levels of therapeutic antivenin after administration, and to assist in the development of a more effective treatment regimen. There have been some studies reporting the use of the ELISA to measure the amount of venom in the serum of human snakebite patients,4-10 but only one of these studies was done for snakebite patients in the United States. In 1987, Minton10 reported the use of an ELISA to determine the presence of pit viper venoms in various specimens from human snakebite cases in the United States. In this study, he reported testing the urine from one person, but no venom was detected.

Studies that measured the amounts of circulating therapeutic antivenin after adminstration to a snakebite patient are even more rare. One study done in Brazil suggested that too much antivenin was being administered.11 This conclusion was based on the finding that blood antivenin levels were still high after the envenomation had subsided. There have been no published studies on the blood levels of therapeutic antivenin after administration to a snakebite victim in the United States. We report here results from the measurement by ELISA of serum levels of therapeutic antivenin administered to an individual bitten by a western diamondback rattlesnake (*Crotalus atrox*) and the detection of venom in the urine.

CASE REPORT

The patient was a white man who was bitten on the left thumb by a western diamondback rattlesnake

(*Crotalus atrox*) that was part of his private collection. Identification of the snake was confirmed by one of us (R.A.). The bite occurred between 12:30 am and 2:30 am, and the patient arrived at the hospital emergency room at 3:15 am. On admission, blood and urine samples were collected, the patient's blood was cross-matched, and a complete blood profile was done. Photographs of the hand and arm were taken. The patient was treated with 10 vials (a total of 100 mL) of Antivenin (Crotalidae) Polyvalent (Wyeth-Ayerst). The first four vials (total of 40 mL) were administered by intravenous push. Over the next 4 hours, two and then four more vials of antivenin were given in about 500 mL of physiologic saline. A methylprednisolone (Medrol) dose pack was started and silver sulfadiazine (Silvadene) ointment was applied to the local lesion. The patient was discharged from the hospital on the third day after the bite and was seen for follow-up on the sixth day, as well as at 1 month, 6 weeks, 10 weeks, and 20 weeks after the bite. Serum and urine samples were taken at follow-up visits.

Enzyme-Linked Immunosorbent Assays (ELISAs)

Microtiter plates (Corning nonsterile 96-well, flat bottom polystyrene) were coated overnight at 4 degrees Celcius with *Crotalus atrox* venom in coating buffer (0.1M Na2CO3/NaHCO3 buffer, pH 9.6) at a concentration of 1 µg/mL (detection of therapeutic antibody) or various dilutions of antivenin (detection of venom). A volume of 200 µl/well was used throughout all assays. Between incubations, plates were washed (3X) in 0.15M NaCl containing 0.05% polysorbate (Tween). A blocking step using 2% BSA in incubation buffer (PBS-Tween) for 1 hour at room temperature was used before addition of samples. Rabbit antihorse IgG or goat antirabbit IgG labeled with alkaline phosphatase (Sigma Chemical Co, St. Louis, Mo) was used for 1 hour at 37¡C at a dilution of 1:1,000 in incubation buffer. After the last washing step, the substrate (p-nitrophenyl phosphate disodium, Sigma Chemical Co) in 1M Tris buffer containing 3 mM MgCl2, pH 9.8, was added and the absorbance at 405 nm was measured using a Molecular Devices ELISA plate reader.

Measurement of Therapeutic Antivenin Levels

The ELISA used to assay serum samples for their content of therapeutic antivenin was essentially as described. Blood samples were collected without anticoagulant, and the serum was separated and stored at -20 degrees Celcius until assayed. Before assaying the serum samples, preliminary assays were done to determine the best antigen (venom or venoms) to use for detection of therapeutic antivenin. Four venoms are used as immunogens in the production of the therapeutic antivenin (*Crotalus atrox*, *C adamanteus*, *C durissus terrificus*, and *Bothrops atrox*), and the serum of interest came from a patient known to have been bitten by the western diamondback rattlesnake (*C atrox*). Thus, it was necessary to determine whether *C atrox* venom alone or a mixture of all four venoms would be the optimum antigen to use for detection. Microtiter plates were coated with either *C atrox* venom (1 µg/mL) or a mixture of the four venoms at a final concentration of 1 µg/mL. The assay was done as described. Therapeutic antivenin (Antivenin [Crotalidae] Polyvalent) at different dilutions in incubation buffer was added and incubated at 37 degrees Celcius for 1.5 hours. Antihorse IgG conjugated with alkaline phosphatase at various dilutions was added. It was determined that coating with *C atrox* venom alone yielded the most sensitive ELISA; therefore, this procedure was used for the remainder of the assays in the study.

For assaying the patient's serum for therapeutic antivenin, *Crotalus atrox* venom was used to coat the plates, and the procedure was the same as described. Test samples, antivenin (lot No. CX13AZ) standards (ranging from 1:10 to 1:160), and normal human serum (negative control) were diluted in 1% BSA in incubation buffer and added in duplicate to the wells. The remainder of the assay was the same as described. Other controls included coated wells that received no sample, wells that received no conjugated antibody, and wells that received incubation buffer only.

Measurement of Venom Levels

The ELISA was also used to measure the amounts of *C atrox* venom in serum and urine at different

times after the envenomization. Urine samples were frozen at -20 degrees Celcius until used. For these assays, plates were coated with a monovalent rabbit antivenin to *C atrox* venom produced in the Venom Research Laboratory at Oklahoma State University, as previously described by Ownby and Colberg,12 diluted 1:400. Antihorse IgG conjugated with alkaline phosphatase (dilution of 1:1,000) was added at 37 degrees Celcius for 1 hour. Washing steps and detection with substrate were as described. *Crotalus atrox* venom dilutions in incubation buffer were assayed on each plate as a positive control and to prepare a standard curve for venom. Control human serum or urine at the same dilutions as the test samples was used as a negative control.

RESULTS AND DISCUSSION

Figure 1 shows the determination of the levels of therapeutic antivenin in the serum at various times after administration. The first measurement was made on serum taken before antivenin administration, and the absorbance level was below 0.100. The level rose quickly and remained high until the fifth day after administration. Between 5 and 30 days after administration, he decline in antivenin levels was more gradual, then stayed essentially the same until day 45. Low levels of antivenin were detectable at 46 days and even 4 months after administration. These results are consistent with those of the three previously published studies in which levels of therapeutic antivenin were measured.11,13,14 In one study, the levels of monospecific antivenins were measured in patients bitten by the Malayan pit viper, *Calloselasma rhodostoma*, and with all three antivenins used, the plasma levels declined rapidly over the first 10 hours and then decreased more gradually over the next 90 hours.14 The first part of this biphasic decline was attributed to the formation of venom-antivenin complexes and possibly some tissue distribution. The second part of the decline was attributed to elimination of the antivenin. The investigators found that in 8 of 26 patients the venom levels in the plasma rose during this time, indicating that perhaps more antivenin should have been given. In a different study, serum samples from patients bitten by various *Bothrops* species were analyzed for their content of therapeutic antivenin.11 Again, a rapid decline in antivenin levels was observed in the first few hours after administration, followed by a slower decline over the next 3 days, with complete elimination by 40 to 50 days after administration. In this study, the blood levels of venom did not increase after administration of antivenin, and the authors indicated that the doses of antivenin were excessively high. All of these studies, including ours, indicate that the ability to measure the blood levels of therapeutic antivenin and venom could be valuable in assisting the attending physician in decisions about whether more antivenin is required for neutralization of the venom.

Figure 2 shows the determination of *Crotalus atrox* venom in this patient's urine. Venom was not detectable above background until the fourth day after the bite, and it was still measurable 6 days after the bite. An absorbance value of 0.140 was determined from the standard curve (not shown) to represent approximately 20 ng/mL of venom in the urine. These results are consistent with those of other studies in which the presence of venom in the urine was determined.5,9 In follow-up of patients bitten by the Malayan pit viper, Ho et al5 found detectable amounts of venom in the urine in 88% of the patients on admission, but they did not measure urine venom concentrations after that. In a study of venom levels in patients bitten by European vipers,9 an ELISA was developed to measure venom in the urine, but no urine levels were reported in the paper. The same group of investigators previously reported that venom levels in the urine are similar to those measured in the serum and that a lower background was obtained in the assay of urine than in that of serum.8 We also found this to be the case, and therefore we are not reporting any serum venom levels in this case. Audebert et al8 also suggested that the concentration of venom in the urine might be a better measure of the severity of the snakebite poisoning but cautioned that the levels of venom in the urine might depend on the volume of urine in the bladder at the time of sampling.

In the only report of measurement of the levels of pit viper venoms in humans in the United States,10 urine was tested from one patient, and it was found to be negative using antibody conjugates prepared against the venoms of *Crotalus atrox*, *C scutulatus*, and *Agkistrodon contortrix*. The author explained that the negative result could be because of failure of the test procedure or

because of the manner of specimen collection, but no details are given. He did discuss the importance of improving the sensitivity and specificity of the ELISA used for such studies.

In conclusion, we report here for the first time the levels of therapeutic antivenin in the serum and the level of venom present in the urine of a human bitten by a western diamondback rattlesnake (*Crotalus atrox*). These data indicate that therapeutic antivenin levels can be measured and that the venom, at least in this case, first appeared in the urine 4 days after the bite.

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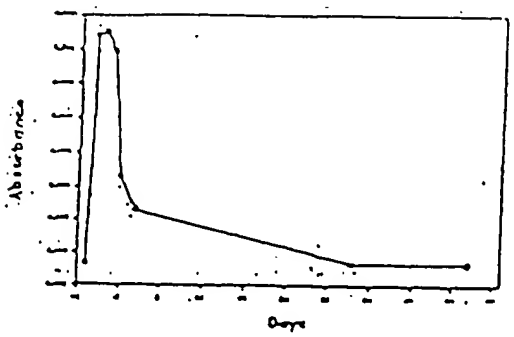
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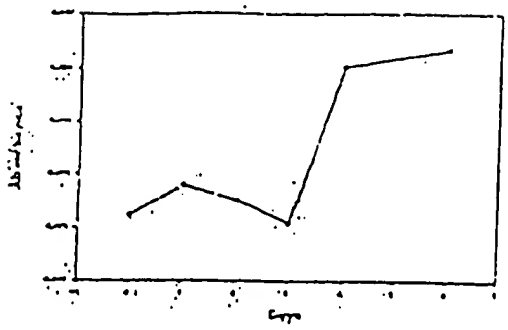

Toxicon, Vol. 26, No. 5, pp. 491–499, 1988
Printed in Great Britain.

0041-0101/88 $3.00 + .00
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STRONGLY ENHANCED TOXICITY OF THE MUSHROOM TOXIN α-AMANITIN BY AN AMATOXIN-SPECIFIC FAB OR MONOCLONAL ANTIBODY

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(*Accepted for publication* 17 *November* 1987)

H. FAULSTICH, K. KIRCHNER and M. DERENZINI. Strongly enhanced toxicity of the mushroom toxin α-amanitin by an amatoxin-specific Fab or monoclonal antibody. *Toxicon* 26, 491–499, 1988. — A monoclonal antibody, with high affinity against the mushroom toxin α-amanitin, was prepared. Administration of the Fab fragment of the monoclonal antibody to mice caused a 50-fold increase in α-amanitin toxicity. Electron micrographs showed normal appearance of hepatocytes but typical, amanitin-induced lesions in cells of the proximal convoluted tubules of the kidney. The pronounced nephrotoxicity is mainly explained by glomerular filtration and tubular reabsorption of the Fab-amatoxin complex and, to a lesser extent, of the immunoglobulin–amatoxin complex, which is still c. twice as toxic as free α-amanitin. To our knowledge this is the first reported case where immunoglobulins or their fragments enhance rather than decrease the activity of a toxin. Accordingly, immunotherapy of Amanita mushroom poisoning in humans does not appear promising.

INTRODUCTION

EARLY ATTEMPTS at using serum to reverse mushroom toxicity date back to the beginning of this century (DUJARRIC DE LA RIVIERE, 1929). At that time a horse was 'immunized' with crude extracts of *Amanita phalloides*, but the efficacy of the serum was never clearly proven. In retrospect it seems unlikely that native amatoxins, peptides of c. 900 mol. wt. would exhibit any immunogenic activity. Antibodies against amatoxins can be obtained when the peptides are conjugated with proteins. However, amatoxins bound to proteins are extremely poisonous for animals (CESSI and FIUME, 1969; DERENZINI *et al.*, 1973; ONETTI *et al.*, 1974). Alpha- and β-amanitin were tolerated, however, when attached to a glycoprotein, fetuin, as a carrier. The sera obtained were mainly used for diagnostic purposes (FAULSTICH *et al.*, 1975, 1982; FIUME *et al.*, 1975; FAULSTICH, 1984).

Rabbit antibodies have been assayed also for their immunotherapeutic efficacy (KIRCHNER and FAULSTICH, 1986). It was shown that a purified fraction of the polyclonal immunoglobulins was of no therapeutic value in mice, but rather increased the toxicity of α-amanitin by a factor of two. In the present study we describe the preparation of a monoclonal antibody against α-amanitin, and an approach to immunotherapy using this immunoglobulin or its Fab fragment.

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MATERIALS AND METHODS

[^{3}H]-6-*O*-methyl-dehydroxymethyl-α-amanitin (spec. activity = 7.4 Ci/mmole) was prepared in our laboratory (FAULSTICH *et al.*, 1975). The purity of the labeled compound was ascertained by its typical U.V. spectrum and by thin layer chromatography. On silica (HF^{254}, Merck, Darmstadt) developed with chloroform (65) methanol (25) water (4) it represented a single spot detectable by U.V. absorption. Scraped off and eluted with methanol the extract contained > 90% of the total radioactivity applied to the silica plate.

Beta-amanitin was prepared from aqueous extracts of *Amanita phalloides* mushrooms by chromatographic procedures (FAULSTICH *et al.*, 1973), performed on a preparative scale. The toxin was coupled to fetuin (Sigma, Munich) as described previously (KIRCHNER and FAULSTICH, 1986). The molecular ratio of amatoxin attached to the protein as determined by spectrophotometry (ε_{305} = 13,500 $M^{-1}cm^{-1}$) was 1.8. Aliquots containing 25 μg amatoxin were mixed with Freund's complete adjuvant and injected intracutaneously into Wistar rats of 200 g body weight. Two booster injections, each containing 30 μg amatoxin in 0.9% NaCl, were administered i.m. 4 and 20 weeks after the first injection. Blood samples (0.3 ml) taken from the tail vein 3 weeks after each antigen injection were analyzed for their titers of amatoxin-specific antibodies by ELISA.

Enzyme-linked immunosorbent assay (ELISA) was performed according to the method of ENGVALL (1980). The assay used an α-amanitin conjugate of bovine serum albumin (FAULSTICH and FIUME, 1985; FAULSTICH *et al.*, 1983) attached to the walls of microtiter wells (Dynatech, Denkendorf, FRG) as immunoadsorbant, and anti-rat-IgG (of the rabbit) coupled to horseradish peroxidase (Miles, Frankfurt) for detection.

Spleen cells of the immunized rats were fused with mouse myeloma cells (P3-X-63-Ag8-653), and hybridoma colonies were isolated according to the procedure of LOSAT *et al.* (1981). Colonies producing amatoxin-specific antibodies were sub-cloned by the limiting dilution technique (GOODING, 1980). A selected amatoxin-specific hybridoma clone was raised in nude mice. Ascites fluid was harvested after 10 to 20 days and the monoclonal antibody was purified by affinity chromatography on Protein-A-Sepharose CL 4B (Pharmacia, Uppsala) (MCGREGOR *et al.*, 1983). The immunoglobulin was characterized by immunodiffusion (OUCHTERLONY, 19[illegible] using isotype-specific antisera (Miles, Frankfurt). Fab-fragments were obtained (ROUSSEAUX *et al.*, 1983) by digestion with mercuripapain. The Fab-fragment was separated from Fc-fragment and undigested immunoglobulin by passage through a Protein-A-Sepharose CL 4B column. Spectrophotometry of the amanitin-immunoglobulin complex and the equilibrium dialysis experiments have been described elsewhere (KIRCHNER and FAULSTICH, 1986).

Female mice (NMRI) of 18 – 25 g body-weight were poisoned by i.p. administrations of various doses of α-amanitin. About one min after the toxin injection the animals received i.v. equivalent amounts of monoclonal IgG or Fab. All protein fractions contained 0.9% NaCl and were sterilized by ultrafiltration immediately before use. A group of mice received various doses of α-amanitin i.v. complexed with IgG. The complex was prepared by incubating 1 equivalent of IgG with 2 equivalents of α-amanitin 1 hr before administration to the animals.

For electron microscopy, kidney samples were fixed, immediately after animal sacrifice, in 2.5% glutaraldehyde in 0.1 M Sorensen buffer, pH 7.2 and post-fixed in 1% OsO_4 in the same buffer. After alcohol dehydration, the samples were embedded in Epon. Ultrathin sections were stained with uranium and lead

RESULTS

Preparation of the monoclonal antibody and its Fab fragment

Ascites fluid of nude mice contained up to 7 mg/ml amatoxin-specific IgG. Because of the absence of host-specific immunoglobulins the antibody was easily purified by affinity chromatography on Protein-A-Sepharose (MCGREGOR *et al.*, 1983). In SDS – gel electrophoresis the purified immunoglobulin appeared as one heavy chain and one light chain (Fig. 1).

The subclass of the monoclonal IgG was determined by immunodiffusion (OUCHTERLONY, 1968), and found to be IgG2a. Elution of an IgG2a from an affinity column required a glycine/HCl buffer of pH 3, and although it was neutralized immediately the treatment may have caused partial denaturation of the globulin. Spectrophotometric examination of the binding capacity (KIRCHNER and FAULSTICH, 1986) showed, however, that the binding ratio of α-amanitin to immunoglobulin was 1.92 : 1, which is close to the expected value of 2 : 1.

For preparation of the Fab fragments the IgG2a was digested with mercuripapain. The reaction was complete after 2 – 3 hr as shown by the appearance of only two bands in SDS – PAGE corresponding to Fab and Fc fragments. As illustrated in Fig. 2, undigest[illegible]

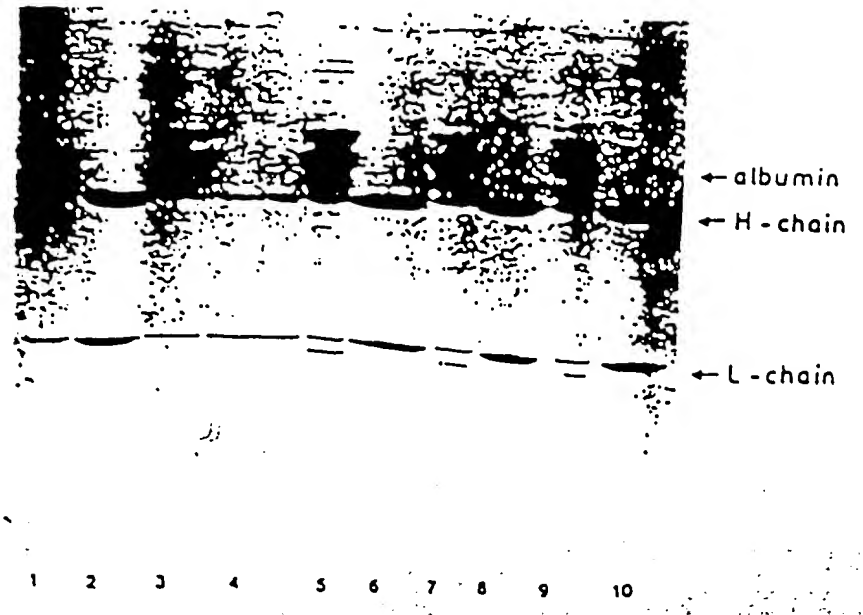

FIG. 1. AFFINITY PURIFICATION OF A MONOCLONAL IMMUNOGLOBULIN FROM THE ASCITES OF NUDE MICE AS FOLLOWED BY SDS – PAGE.
Lane 1, 3, 5, 7, 9: crude ascites of 5 animals (the major band is mouse albumin). Lane 2, 4, 6, 8, 10: the monoclonal immunoglobulin fractions after purification. Only the heavy (H-) and light (L-) chain of the IgG are visible.

heavy chain was still visible after 1 hr, while after 4 hr of digestion side products were detected. Again, Protein-A-Sepharose was used to separate Fc fragments and unmodified IgG from Fab, which was obtained in ca. 90% yield.

The affinity of the monoclonal IgG2a and its Fab fragment for amatoxins was assayed by equilibrium dialysis experiments (KIRCHNER and FAULSTICH, 1986), using a tritium-labeled amatoxin, [^{3}H]-6'-*O*-methyl-dehydroxymethyl-α-amanitin. In 8 experiments the labeled amatoxin derivative exhibited a mean dissociation constant (K_D) of 4.0 ± 0.2 nM (graph not shown). In similar experiments the Fab fragment showed a K_D value of 3.8 nM, and was thus the same within the limits of error.

Toxicological studies

In NMRI mice used for the toxicological experiments, α-amanitin showed an LD_{50} value of 0.75 mg per kg body weight. The toxin was administered i.p., which gave the same LD_{50} value as that given by i.v. administration. The animals died from liver dystrophy, from the third day onwards.

When mice were poisoned i.p. with an LD_{50} of α-amanitin, and treated subsequently i.v. with an equivalent amount of monoclonal IgG, or Fab, all animals died, some of them earlier than the controls. This suggested to us that the antibody and the Fab had not decreased but rather enhanced the toxicity of α-amanitin. Indeed we showed that the LD_{50} of α-amanitin in the presence of its immunoglobulin was 0.39 mg per kg body weight, corresponding to a 2-fold increase of toxicity. A much greater increase of toxicity occurred when the poisoned mice were treated with Fab. In this case we determined an

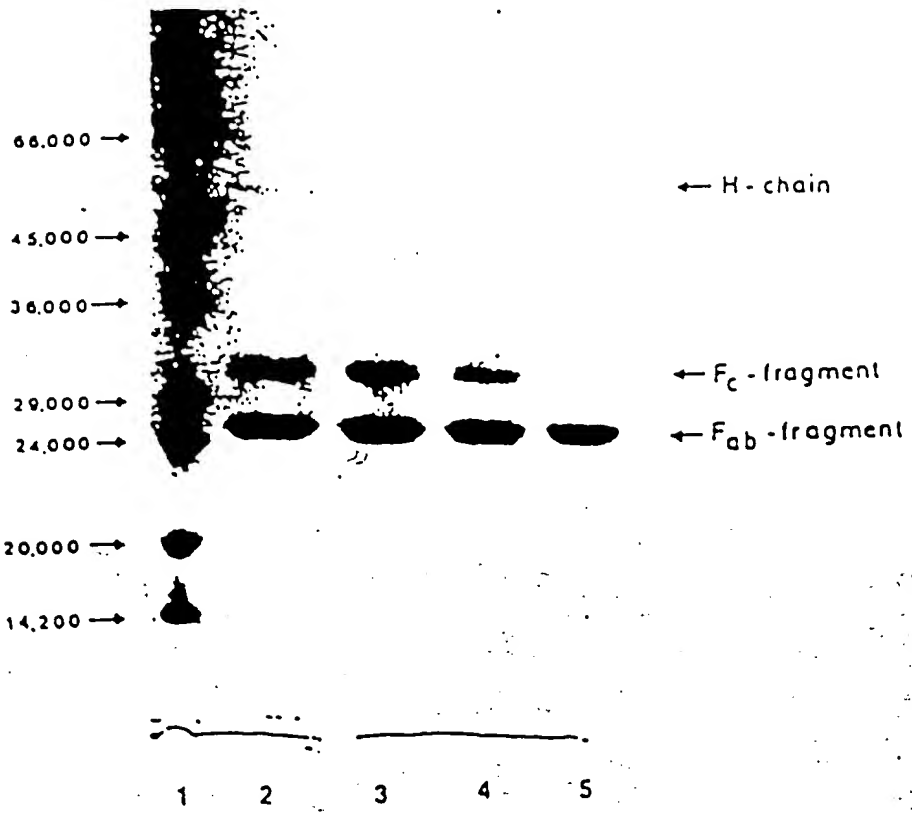

FIG. 2. MERCURIPAPAIN DIGESTION OF THE MONOCLONAL ANTIBODY AS MONITORED BY SDS – PAGE. Lane 2 – 5: reaction mixture after incubation with the enzyme for 1, 2, 3 and 4 hr. Lane 1: marker proteins: bovine serum albumin, 66,000; ovalbumin, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin inhibitor, 20,000; α-lactalbumin, 14,200.

LD_{50} of 0.015 mg per kg body weight, which corresponds to a roughly 50-fold increase in toxicity (Table 1).

In order to identify the preferred target of α-amanitin under these conditions, livers and kidneys of the animals were examined by electron microscopy. In the Fab-treated mice the hepatocytes appeared completely normal (not shown), while considerable nuclear lesions were found in the proximal convoluted tubule cells of kidney (Fig. 3a, b). Since the nuclear lesions appeared as soon as 2 hr after poisoning we conclude that damage of the kidney tubule cells was the primary toxic event. According to this finding most of the animals are likely to have died from kidney failure, due to severe necrosis of the proximal convoluted tubules.

As with the Fab-treated mice, the animals treated with the whole immunoglobulin exhibited nuclear lesions predominantly in the kidney. These lesions occurred later than in the Fab-treated mice, but after 6 hr most of the nuclei of the proximal convoluted tubule cells had changed their structure. As in the Fab-treated animals the hepatocytes of the immunoglobulin treated mice appeared normal. There were, however, scattered nuclear lesions in some of the sinusoidal cells. After 48 hr the kidney cells of the immunoglobulin-treated mice had developed a severe necrosis.

TABLE 1. SENSITIVITY OF NMRI MICE (FEMALE, 18 – 25 g) TO VARIOUS i.p. DOSES OF α-AMANITIN IN THE PRESENCE OR ABSENCE OF AMATOXIN-SPECIFIC IMMUNOGLOBULIN OR FAB-FRAGMENT

α			α/IgG2a			α/Fab		
Dose mg/kg	Mortality	Days of survival	Dose mg/kg	Mortality	Days of survival	Dose mg/kg	Mortality	Days of survival
1.00	8/8	3 – 4	0.59	6/6	3 – 16	0.80	4/4	1
0.81	12/13	3 – 4	0.51	4/4	2 – 4	0.40	6/6	1 – 5
0.76	3/6	3 – 4	0.49	6/6	2 – 5	0.20	4/4	3 – 5
0.59	3/20	3 – 4	0.29	0/4	–	0.10	5/5	4
0.41	0/10	–				0.05	4/4	4 – 5
						0.02	10/10	4 – 5
						0.01	2/9	4 – 5
						0.005	0/4	–

(α): α-amanitin, (α/IgG2a), (α/Fab): α-amanitin with subsequent i.v. administration of IgG2a (half molar amount) or Fab (molar amount).

Mortality = number of animals died/number of animals treated.

All protein fractions were in 0.9% NaCl and sterilized by ultrafiltration immediately before use.

In general, the monoclonal immunoglobulin as well as its Fab fragment had protected the hepatocytes of mice from amanitin toxicity, but had caused severe amanitin lesions in the cells of the proximal convoluted tubules, leading, in both cases to an enhanced toxicity and to death by kidney failure.

DISCUSSION

Toxicity in mice of α-amanitin (i.p.), followed by i.v. administration of a monoclonal antibody, is very similar to the toxicity caused by i.v. administration of the amanitin-immunoglobulin complex, as indicated by the same LD_{50} value and a similar course of intoxication (data not shown). Presumably the toxin when administered i.p. is trapped by the immunoprotein soon after entering blood circulation.

Trapping by immunoglobulins can decrease the filtration rate and thus retard renal excretion of small molecules as shown, for example, for digoxin (SCHMIDT *et al.*, 1974). This effect may enhance the toxicity of low mol. wt compounds, and it cannot be excluded that such an effect contributes to the higher toxic activity of α-amanitin in the presence of its immunoproteins. However, more important for the increased toxicity is certainly that complexing amatoxins to Fab or immunoglobulin changes their target cells.

Free amatoxins cause lesions predominantly in the parenchymal cells of the liver. In contrast, when amatoxins are covalently linked to albumin, or other proteins, they mainly affect cells involved in the protein turnover of the organism (DERENZINI *et al.*, 1974), such as macrophages, sinusoidal cells of the liver and protein-absorbing cells of kidney. Damage to these kinds of cells leads to markedly enhanced *in vivo* toxicity, (for a review see FAULSTICH and FIUME, 1985). As shown in the present study amatoxins, when administered together with their immunoproteins have an *in vivo* toxicity much higher than free amatoxins. In addition, they do not affect hepatocytes but those cells of liver and kidney which are largely involved in the turnover of proteins. We therefore conclude that the immunocomplexes of α-amanitin exhibit toxic activities very similar to amatoxins covalently linked to proteins.

While amatoxins conjugated with proteins mainly affect macrophages and sinusoidal cells, the immunocomplexes of amatoxins exhibit specificity for kidney cells. Nuclear lesions found in the cells of the proximal convoluted tubules are shown in Fig. 3. They

FIG. 3a. MOUSE PROXIMAL CONVOLUTED TUBULE CELL. 2 hr AFTER ADMINISTRATION OF 0.02 mg/kg BODY WEIGHT AMANITIN PLUS THE EQUIMOLAR DOSE OF FAB.
The nucleus of the tubule cell shows a marked condensation and margination of chromatin and a clustering of ribonucleoprotein structures (arrows). BB, brush border. Bar, 1 μm.

include fragmentation and segregation of ribonucleoprotein components, chromatin clumping, clustering of interchromatin-like granules and accumulation of perichromatin-like granules. These changes correspond to the lesions induced by free amatoxins in the hepatocytes of mouse and rat (MARINOZZI and FIUME, 1971).

The occurrence of nuclear lesions in kidney tubule cells indicates that complexes of immunoproteins and amatoxins are subject to glomerular filtration and tubular reabsorption. This is not self-evident, because the filtration rate of proteins decreases with increasing mol. wt. Albumin (66,000 mol. wt), for example, has a glomerular sieving coefficient of only 0.0001, (BALDAMUS *et al.*, 1971) and the filtration rate of immunoglobulins (155,000 mol. wt) is even lower (ROVIRA-HALBACH *et al.*, in press). Nevertheless filtration and reabsorption of the amanitin-immunoglobulin complex must be high enough to produce after several hr a toxin accumulation in the proximal convoluted tubule cells which causes kidney failure and finally death of the animals.

Fab fragments are filtered much more efficiently than immunoglobulins. With a mol. wt of 45,000 they resemble Bence–Jones proteins (44,000), which have a glomerular sieving coefficient of 0.09 (MAACK *et al.*, 1985). Accordingly, Fab fragments do

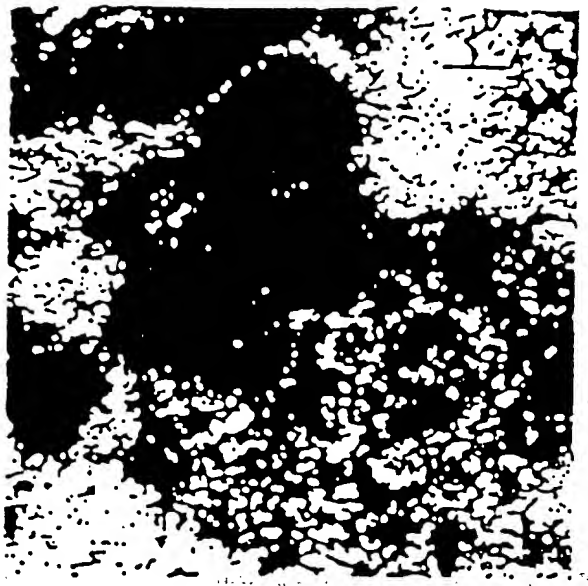

FIG. 3b. DETAIL OF FIG. 3a SHOWING THE CLUSTERED RIBONUCLEOPROTEIN STRUCTURES F, fibrillar nucleolar fragments; G, nucleolar fragment with segregated fibrillar and granular components; IG, interchromatin granules. Bar, 0.2 μm.

retard urinary excretion of small molecules as do immunoglobulins (BUTLER *et al.*, 1977). The high filtration rate of the Fab-amanitin-complex followed by extensive reabsorption in the tubules is clearly the crucial cause of the 50-fold increase of α-amanitin toxicity.

The present study was prompted by reports that high-affinity antibodies raised against digoxin were able to reverse the toxic effects of the drug in animals (CURD *et al.*, 1971; SCHMIDT and BUTLER, 1971; ZALGBERG *et al.*, 1983; SMITH *et al.*, 1979; BUTLER *et al.*, 1977). Moreover, Fab fragments of the digoxin-specific antibodies were successfully employed in a case of human suicidal digoxin poisoning (SMITH *et al.*, 1976). Such beneficial effects were not observed with the amatoxin-specific immunoproteins. This failure is most probably explained by the different locations of the targets of the two toxins. Incorporation of digoxin into tubule cells prevents the glycoside from binding to its target enzyme, the Na^+/K^+-dependent ATPase located on the outer surface of plasma membranes. Alpha-amanitin, on the other hand, when complexed with its Fab, apparently uses the protein as a vehicle for penetration into tubule cells thereby gaining access to its target enzyme, the DNA-dependent RNA polymerase II (or B) (STIRPE and FIUME, 1967). Thus, the toxic activity of digoxin can be expected to be decreased, while that of α-amanitin may be greatly enhanced.

In conclusion, the present results speak against the feasability of an immunotherapy of human *Amanita* poisoning using a monoclonal antibody or its Fab fragment. They confirm our previous finding that purified polyclonal antibodies of rabbit enhance amanitin toxicity in the mouse by a factor of two (KIRCHNER and FAULSTICH, 1986). On the other hand, the Fab-treated mice died from very low doses of α-amanitin indicative of a highly specific targeting of the toxin to the proximal convoluted kidney cells. It is

therefore possible that the α-amanitin/Fab-treated mouse could be employed as a model for studying acute kidney failure.

Acknowledgements — This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

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THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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Utilization of Antidrug Antibody Fragments for the Optimization of Intraperitoneal Drug Therapy: Studies Using Digoxin as a Model Drug

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Accepted for publication October 27, 1993

ABSTRACT

The direct administration of chemotherapeutic agents into the peritoneal cavity has been investigated as a method to treat cancers residing within the peritoneum. The benefits of i.p. drug administration are limited, however, by the systemic toxicity of antineoplastic drugs which diffuse out of the peritoneum and into the general circulation. We propose that antidrug antibody fragments may be useful in binding chemotherapeutics in the general circulation, thereby reducing the systemic tissue exposure and toxicity resulting from such i.p. therapy. Inasmuch as antibody fragments directed against antineoplastic agents are not available, we tested our hypothesis by using i.v. administered ovine anti-digoxin Fab fragments and determined their ability to limit digoxin tissue exposure and toxicity in mice after an i.p. digoxin injection. The rate of digoxin disappearance from the peritoneal cavity and the fraction of digoxin unbound in the peritoneal cavity were also assessed to determine the effect of the antibody fragments on peritoneal exposure. Our results showed that the antidigoxin antibody fragments can greatly decrease digoxin tissue exposure and toxicity without affecting peritoneal exposure, unbound fraction of digoxin in the peritoneum or peritoneal digoxin disappearance rate. Although the utility of drug-binding antibodies and antibody fragments for the treatment of drug intoxication is well known, these results demonstrated the potential ability of antidrug antibody fragments to improve the site-specificity of drug therapy.

Direct drug delivery into the peritoneal compartment has been studied as a method to treat cancers residing within the peritoneum. It has been theorized that i.p. administration of cancer chemotherapeutic agents would allow for greater drug exposure to the tumor while simultaneously decreasing systemic exposure and toxicity (Dedrick *et al.*, 1978; Clay and Howell, 1992). Unfortunately, results of early phase I and phase II clinical studies assessing the benefits of i.p. delivery have shown lower than optimal decreases in toxicity or increases in effect (Howell, 1988; Clay and Howell, 1992).

One reason for the lack of success of direct i.p. therapy for some drugs is the development of toxicity arising from the distribution of the drug from the peritoneal cavity into the general circulation. An approach to overcome this problem is to administer drug into the peritoneum and simultaneously a drug-complexing agent into the systemic circulation, so as to reduce the exposure of systemic tissues to free drug. Howell *et al.* (1982) have investigated this optimization approach in a study of the utility of sodium thiosulfate for the reduction of cisplatin toxicity. The benefit of this combined therapy, however, was limited by the slow rate of thiosulfate-cisplatin complexation in plasma (Howell, 1988).

We postulate that antidrug antibody fragments may be superior complexing agents for this approach. Antibody fragments may be produced against a wide variety of substances, including many drugs. Antibodies generally exhibit extremely high specificity and binding affinity for their antigen (K_a common range: 10^7 to 10^{10} M^{-1}), as well as rapid binding rates (commonly: 10^6 $M^{-1} \cdot sec^{-1}$) (Stryer, 1988). In addition, the high molecular weight and polar characteristic of antibody fragments should result in favorable distribution properties (*i.e.*, the antibodies are expected to remain primarily in plasma, with a very slow diffusion rate across the peritoneal membrane).

To determine the potential use of antidrug antibody fragments for the optimization of i.p. drug delivery, we have conducted a preliminary investigation into the general pharmacokinetic and physiological implications of the approach, by using the cardiac glycoside digoxin and ovine antidigoxin Fab antibody fragments (Digibind, Burroughs-Wellcome Company, Re-

Received for publication July 2, 1993.
[1] A Schering-Plough Pre-Doctoral Fellow.

ABBREVIATIONS: AUC_T, area under the concentration vs. time curve; AUC_P, area under the peritoneal concentration vs. time curve; FE_T, fractional tissue exposure; FE_P, fractional peritoneal exposure; FRIA, fluorescence polarization immunoassay; $Fu(p)$, fraction of digoxin unbound in the peritoneal cavity.

search Triangle Park, NC) as model agents. Use of a model system was necessitated because antibody fragments directed against commonly used cancer chemotherapeutic agents are not yet available.

The present study was designed, therefore, to determine whether the simultaneous administration of digoxin (i.p.) and antidigoxin antibody fragments (i.v.) would: 1) reduce observable digoxin toxicity; 2) reduce systemic digoxin tissue exposure; 3) affect the unbound fraction of digoxin within the peritoneal cavity; and 4) affect the disappearance rate of digoxin from the peritoneal cavity, when compared to administration of digoxin (i.p.) alone.

In addition, computer simulations were conducted to predict the ability of the antibody fragments to affect digoxin tissue and peritoneal exposure. The results of these simulations were used to determine an appropriate antibody fragment dose to minimize tissue exposure while not affecting peritoneal exposure. Further simulations were conducted to assess the influence of the antibody fragment-digoxin association rate, dissociation rate and affinity on tissue exposure. Simulations were carried out by using the model depicted in figure 1 through the use of ADAPT II pharmacokinetic software (D'Argenio and Schumitzky, 1992).

Methods

Simulations. The pharmacokinetic model used to describe digoxin disposition in the presence of drug binding antibody fragments is shown as figure 1 and is based on the following equations:

$$\frac{dC_{dP}}{dt} = -(k_e \cdot C_{dP}) - (k_{on} \cdot C_{dP} \cdot C_{aP}) + (k_{off} \cdot C_{daP}) \quad (1)$$

$$\frac{dC_{aP}}{dt} = \frac{CL_{Da} \cdot C_{dT}}{V_P} - \frac{CL_{Da} \cdot C_{aP}}{V_P} - (k_{on} \cdot C_{dP} \cdot C_{aP}) + (k_{off} \cdot C_{daP}) \quad (2)$$

$$\frac{dC_{daP}}{dt} = \frac{CL_{Da} \cdot C_{daT}}{V_P} - \frac{CL_{Da} \cdot C_{daP}}{V_P} + (k_{on} \cdot C_{dP} \cdot C_{aP}) - (k_{off} \cdot C_{daP}) \quad (3)$$

$$\frac{dC_{dT}}{dt} = \frac{k_e \cdot C_{dP} \cdot V_P}{V_{dT}} - \frac{CL_{Da} \cdot C_{dT}}{V_{dT}} - \frac{CL_{Dd} \cdot C_{dT}}{V_{dT}}$$

$$- (k_{on} \cdot C_{dT}) + (k_{off} \cdot C_{daT}) - \frac{k_{on} \cdot C_{dT} \cdot V_P}{V_{dT}} \quad (4)$$

$$\frac{dC_{aT}}{dt} = -\frac{CL_{Da} \cdot C_{aT}}{V_T} - \frac{CL_{Da} \cdot C_{aP}}{V_T} - (k_{on} \cdot C_{aT})$$

$$- \frac{k_{on} \cdot C_{aT} \cdot C_{dT} \cdot V_{dT}}{V_T} + (k_{off} \cdot C_{daT}) \quad (5)$$

$$\frac{dC_{daT}}{dt} = -\frac{CL_{Da} \cdot C_{daT}}{V_T} - \frac{CL_{Da} \cdot C_{daP}}{V_T} - (k_{off} \cdot C_{daT})$$

$$- \frac{k_{on} \cdot C_{aT} \cdot C_{dT} \cdot V_{dT}}{V_T} + (k_{on} \cdot C_{daT}) \quad (6)$$

$$\frac{dC_{dT}}{dt} = \frac{CL_{Da} \cdot C_{aT}}{V_{dT}} - \frac{CL_{Dd} \cdot C_{dT}}{V_{dT}} \quad (7)$$

Where C_{dP}, C_{aP} and C_{daP} are the concentrations of digoxin, free antibody fragments and antibody fragment-digoxin complex in the peritoneal cavity; C_{dT}, C_{aT} and C_{daT} are the concentrations of digoxin, free antibody fragment and antibody fragment-digoxin complex in their respective central distribution volumes; C_{dT} is the concentration of digoxin within the tissue compartment and t is time. Also, k_e, k_{on}, k_{off} and k_{on} are the rate constants for digoxin absorption from the peritoneum, antibody fragment-digoxin association, antibody fragment-digoxin dissociation, elimination of digoxin and elimination of antibody fragments, respectively. CL_{Da} is the distribution clearance of digoxin; and CL_{Da} is the distribution clearance of antibody fragments between the peritoneal and central compartments. V_P, V_{aT}, V_{dT} and V_T are the volumes of distribution of the antibody fragments, the central volume of distribution of digoxin, the tissue volume of distribution for digoxin and the volume of the peritoneal cavity, respectively.

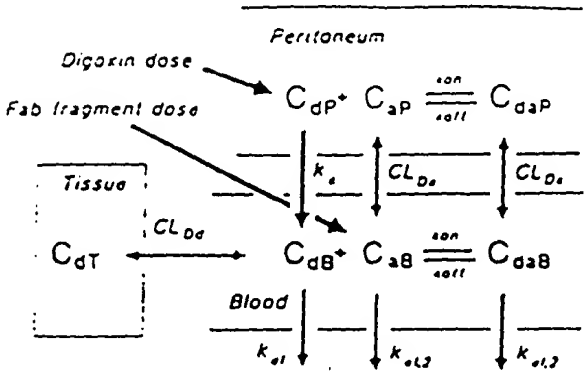

Fig. 1. Pharmacokinetic model used for simulation of the disposition of digoxin and antidigoxin antibody fragments in the mouse. Parameters are described within the text.

Digoxin pharmacokinetic parameters for the mouse (CL_{Dd}, k_e, V_{dT} and V_{dP}) were calculated by conversion of published biexponential data (Griffiths *et al.*, 1984) to data consistent with a two-compartment model. The rate constant k_e was determined experimentally in preliminary experiments. Antidigoxin Fab fragment parameters (V_T and k_{el}) were also taken from Griffiths *et al.* (1984). The elimination and distribution of Fab fragments and the Fab fragment-digoxin complex were assumed to be identical. CL_{Da} was estimated to be 9×10^{-3} liters kg^{-1} min^{-1}.

A series of simulations was conducted to aid in the determination of an appropriate antibody fragment dose for the animal study. In this exercise, the dose of digoxin administered was held at 1.02×10^{-4} mol/kg whereas the antibody fragment dose was increased from 10^{-10} mol/kg to 10^{-3} mol/kg. The association and dissociation rate constants for the antibody fragment were fixed in this series at: $k_{on} = 10^8$ M^{-1} sec^{-1} and $k_{off} = 10^{-1}$ sec^{-1}; producing an equilibrium constant consistent with published values for the antidigoxin Fab fragments (K_{on}: 10^9 M^{-1}; Smith *et al.*, 1970; Curd *et al.*, 1971). The AUC_T and the AUC_P were obtained use the linear trapezoidal method from 0 to 240 min at each dose of the antibody. The FE_T after antibody fragment administration was defined as the AUC_T obtained at each antibody fragment dose divided by the AUC_T observed when digoxin was given without antibody fragment treatment. The FE_P was defined in the same manner.

In a second series of simulations, the effect of altering the antibody fragment association rate, dissociation rate and affinity on FE_T were assessed. The binding parameters were tested in the following ranges: k_{on}, 10^1 to 10^9 M^{-1} sec^{-1}; k_{off}, 10^{-1} to 10^4 sec^{-1}; K_{on}, 10^2 to 10^9 M^{-1}. For these simulations, the doses of antibody fragments and digoxin were held at 1.02×10^{-4} mol/kg. These simulations were conducted to determine which complexation parameter was most important for reducing tissue exposure.

Mortality experiment. Male Balb-C mice (weights: 12.5–23 g; Harlan, Indianapolis, IN) were paired into two weight matched groups ($n = 10$ animals/group). All animals received 8 mg/kg of digoxin (lot no. 03223; Elkins-Sinn, Cherry Hill, NJ) administered by i.p. injection. In the test group of mice, an equimolar dose of antidigoxin ovine immune Fab fragments (512 mg/kg, Digibind, lot no. 8X2748; Burroughs Wellcome) was administered by i.v. injection (via the tail vein) 3 to 5 min before the digoxin dose. Animals were monitored for cessation of respiration and survival time was recorded. The experiment was terminated at 240 min after the digoxin injection and surviving animals were sacrificed by cervical dislocation.

The digoxin injection solution contained 250 μg/ml of digoxin

solubilized in 40% propylene glycol and 10% ethanol. The antidigoxin Fab fragment injection solution contained 40 mg/ml of Fab fragments in distilled water.

Tissue exposure. Male Balb-C mice were randomly separated into a control group (animals receiving i.p. digoxin alone, 800 μg/kg, as a digoxin solution of 25 μg/ml in 45% propylene glycol and 1% ethanol) and a test group (animals receiving i.p. digoxin, 800 μg/kg, and i.v. antidigoxin antibody fragments, 64 mg/kg, as a 10 mg/ml solution in distilled water). The antibody fragment dose used was approximately equimolar to the dose of digoxin administered, assuming a specific binding activity of 50% and a MW of 50,000 daltons for the antidigoxin Fab fragment (Curd et al., 1971; Cano et al., 1992). Three animals from each group were sacrificed by cervical dislocation at 5, 15, 30 and 60 min after injection of digoxin.

Immediately after sacrifice, 5 ml of normal saline was injected into the peritoneal cavity of the expired animal. After massaging the abdomen of the animal, 1 ml of fluid was removed from the peritoneum for analysis of free and total digoxin concentration. The total amount of digoxin in the peritoneal cavity was calculated, assuming a total final peritoneal fluid volume of 5 ml (i.e., it was assumed that the volume added to the peritoneal cavity after sacrifice was very much larger than the peritoneal fluid volume just before sacrifice). The heart, brain and left hind-leg skeletal muscle were also collected for analysis. From these data, the fraction of unbound digoxin in the peritoneum, peritoneal disappearance rate of digoxin and the AUC_Ts were obtained. The AUC_Ts were obtained from 0 to 60 min via the linear trapezoidal method, by using the mean of the three tissue concentrations at each sacrifice time.

Tissue extraction. The method used for extraction of digoxin was modified from that of Berman et al. (1977) for the extraction of digoxin from sheep tissues. Briefly, tissues removed from treated animals were weighed wet, minced with a razor blade, then suspended in 6 ml of normal saline through the use of a Polytron tissue homogenizer (model PT10/35). Digoxin was extracted from the tissue suspension with two washes of 10 ml of methylene chloride. The pooled methylene chloride phases were evaporated to dryness under a stream of nitrogen gas. The extracted digoxin was then reconstituted with normal saline for analysis. The reconstitution volume was varied to prepare samples within the range of analysis specified by the Abbott TDx, i.e., 0.2 to 5 ng/ml.

The efficiency and variability of this method were determined by assessing the extraction of known quantities of digoxin which had been added to prepared tissue homogenates. Five homogenates of mouse heart, skeletal muscle and brain were prepared as above. Digoxin was added directly into the homogenates, which were then treated as described above.

Similarly, the efficiency and variability of extraction of digoxin from the antibody fragments were assessed. Digoxin and antidigoxin Fab fragments were added to a reaction vial in sufficient quantities to produce measurable amounts of total and free digoxin. The contents of the vial were incubated for 90 min at 4°C. Aliquots of 2 ml were removed and subjected to extraction as described above.

Digoxin assay. Digoxin concentrations were analyzed by FPIA with the use of an Abbott TDx automated FPIA device (Digoxin II kits, lot nos. 64094Q100 and 66519Q100; Abbott Laboratories, Abbott Park, IL). As the assay kit was calibrated with human serum standards, a standard curve was constructed with samples in normal saline to avoid inaccuracy due to matrix differences. Correction for matrix differences was accomplished through the use of the following equation obtained from the standard curve: $[digoxin]_{true} = 0.847 \cdot [digoxin]_{assay} - 0.14$ ng/ml. FPIA was chosen as the assay method of choice because of its ability to provide accurate total digoxin concentrations in spite of the presence of low levels of therapeutic antibody fragments (Hansell, 1989; Argyle, 1986).

Ultrafiltration. The free concentration of digoxin in the peritoneal fluid was determined through the use of Centrifree ultrafiltration tubes (lot no. MCA129; Amicon, Beverly, MA). Ultrafiltrated samples were centrifuged at 4000 rpm (approximately 1200 × *g*) for 15 min. A standard curve was generated for assessment of ultrafiltrated samples, as ultrafiltration itself may affect the reported digoxin concentration (Banner et al., 1992). The equation obtained from the standard curve to correct for the combined effect of the normal saline matrix and ultrafiltration was: $[digoxin]_{true} = 0.593 \cdot [digoxin]_{assay} - 0.13$ ng/ml. Ultrafiltration of digoxin by this method was shown previously to provide accurate free digoxin concentrations (Hunsting et al., 1987).

Statistics. Data are presented as mean ± S.D. Significant differences were concluded if $P < .05$, as determined by Student's *t* test, comparing the control (digoxin alone) *vs.* the antibody fragment group (digoxin and antibody fragments administered).

Results

Simulations. The dose-ranging simulations conducted produced a sigmoidal log antibody fragment *vs.* FE_T curve. As expected, the FE_T (the ratio of the digoxin AUC_T obtained after Fab fragment administration relative to control) decreased as the antibody fragment dose was raised relative to the digoxin dose. However, the FE_P remained unchanged in the antibody fragment dose range examined (10^{-10} to 10^{-3} mol/kg, fig. 2). These simulations therefore predicted that dramatic reductions in tissue exposure may be produced at antibody fragment doses which do not decrease peritoneal exposure. At the antibody fragment dose used for the tissue exposure study, 1.02×10^{-4} mol/kg, the simulation produced an AUC_T which was 14.1% of control and an AUC_P which was 100% of control.

The second series of simulations demonstrated that the antibody fragment-digoxin equilibration constant is the binding parameter related most closely to antibody fragment efficacy. FE_T was shown to decrease with increasing antibody fragment affinity (fig. 3); however, paired changes in the association and dissociation rate constants (i.e., k_{on} and k_{off} were altered whereas K_{eq} was held constant) did not affect fractional tissue exposure (data not shown).

Extraction efficiency. The extraction of digoxin from tissues and antidigoxin Fab fragments was of good efficiency and low variability in all cases. Efficiencies for the mouse heart,

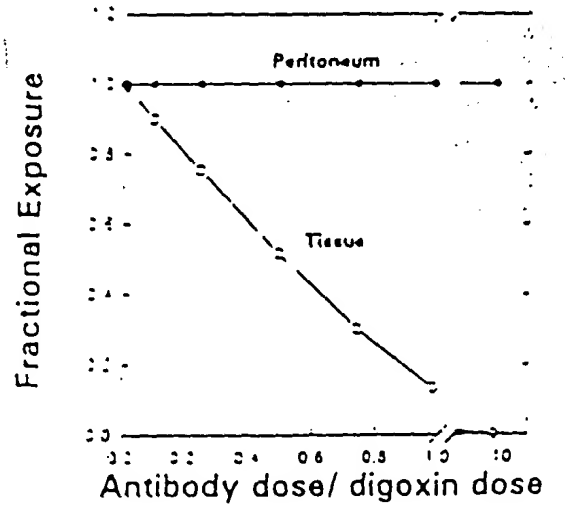

Fig. 2. Simulated fractional exposure of digoxin in the peritoneal (●) and tissue (○) compartments of the mouse, as a function of antibody fragment/digoxin dose ratio. Fractional exposure after antibody fragment administration is obtained by dividing the AUC (0–240 min) at each antibody fragment dose by the AUC (0–240 min) observed when no antibody fragments were given. The dose of digoxin used in this simulation was held at 1.02×10^{-4} mol/kg, whereas the antibody fragment dose was increased from 10^{-11} to 10^{-3} mol/kg. The values of the model parameters used in these simulations were held constant as described in the text.

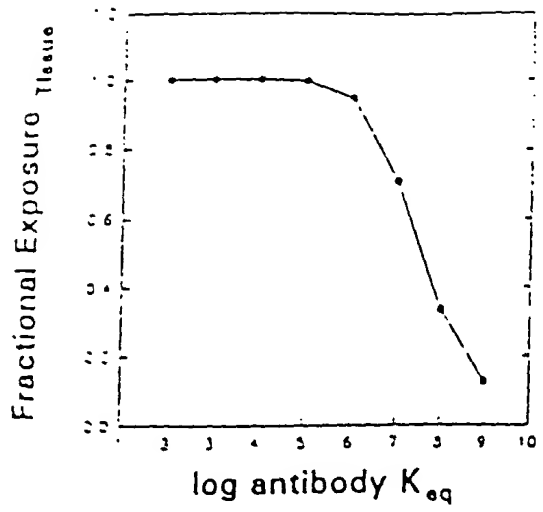

Fig. 3. The simulated relationship of fractional tissue exposure and antibody fragment affinity. The doses of digoxin (i.p.) and antidigoxin Fab fragments (i.v.) were each held at 1.02×10^{-6} M/kg. The range of k_{on} used in these simulations was: 10 to 10^6 M^{-1} sec^{-1}, whereas the range for k_{off} was: 10^{-1} to 10^4 sec^{-1}. Fractional tissue exposure after Fab fragment administration was obtained by dividing the AUC_T (0–240 min) at each Fab fragment dose by the AUC_T (0–240 min) observed when no antibody fragments were given.

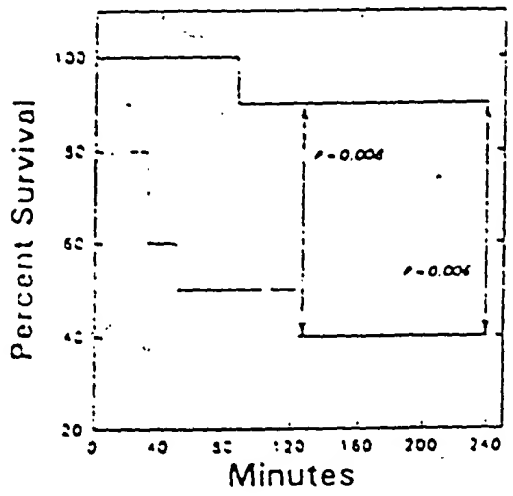

Fig. 4. The percentage of animals surviving a toxic dose of digoxin (8 mg/kg) is shown for animals receiving digoxin alone (···) and those receiving digoxin i.p. and antidigoxin antibody fragments (512 mg/kg) i.v. (——). Mean survival time was used for statistical comparison of the two groups.

skeletal muscle, brain tissue and from the antibody fragments were: 87.0 ± 5.6, 73.2 ± 4.5, 74.7 ± 5.4 and 93.7 ± 1.1%, respectively. Tissue concentrations reported were corrected for these extraction efficiencies.

Mortality. Administration of the antidigoxin fragments greatly increased survival rate. At 240 min after digoxin injection, the survival rate for the antibody group was 90% whereas only 40% of the control group (digoxin alone) survived ($P = .006$). Pretreatment with the antibody fragments decreased mortality substantially (fig. 4).

Tissue exposure. In the three tissues monitored (heart, skeletal muscle and brain), significant reductions in digoxin concentrations were observed (table 1). The AUC_T was reduced substantially in the antibody fragment-treated group relative to the control group. The FE_T, defined as the AUC_T Fab AUC_T control, was 0.56, 0.17 and 0.71 for the heart, muscle and brain, respectively.

Fraction unbound. The Fu_{tp} of the antibody fragment-treated group was not found to be statistically different from the control group at any time (table 1). This finding is supportive of the expected slow rate of diffusion into the peritoneal cavity for the antibody Fab fragment.

Disappearance rate. The rate of disappearance of peritoneal digoxin, which was calculated from the decrease of total peritoneal digoxin with time, was not significantly different for the two groups ($T_{\frac{1}{2}}$ absorption was 12.0 ± 2.0 min for the Fab fragment group and 9.8 ± 1.8 min for the control group). The presence of antibody fragments in the systemic circulation did not appear to influence the rate of digoxin diffusion from the peritoneal cavity. This finding was anticipated because the concentration of digoxin in the peritoneal cavity was expected to be much greater than the free concentration of digoxin in plasma until very late in the experiment when a significant fraction of digoxin has been absorbed. A "sink" condition existed for both the antibody fragment and control groups; therefore, the disappearance rate of peritoneal digoxin was largely unaffected.

The peritoneal exposure to digoxin, which is a function of the disappearance rate of digoxin from the peritoneum and of the Fu_{tp}, was not different for the Fab fragment group compared to the control group.

Discussion

Howell *et al.* (1982) investigated the utility of a competitive binding agent (sodium thiosulfate) for the reduction of the systemic toxicity of an i.p. cisplatin infusion. The investigators simultaneously infused sodium thiosulfate (i.v.) and cisplatin (i.p.) into several ovarian cancer patients, with the expectation that the sodium thiosulfate would bind and neutralize cisplatin immediately as it entered the bloodstream, thereby preventing systemic exposure and toxicity. Unfortunately, it was found that sodium thiosulfate was not able to increase the plasma clearance of free cisplatin significantly, nor was the binding agent able to greatly decrease systemic exposure to cisplatin substantially (Howell *et al.*, 1982). This failure was presumably due to the slow rate of thiosulfate-cisplatin complexation ($T_{\frac{1}{2}}$ = 225 min) relative to the elimination rate of free cisplatin by other means ($T_{\frac{1}{2}}$ = 66 min) (Howell, 1985).

The present study suggests that antidrug antibody fragments may be superior complexing agents for the optimization of i.p. drug delivery. Antibody fragments generally exhibit rapid binding rates, high specificity and affinity, as well as desirable distribution and elimination characteristics. We have investigated the potential use of antibody fragments for the optimization of i.p. drug delivery through a series of experiments and computer simulations. Digoxin and antidigoxin antibody fragments were used as model agents for testing this approach.

Our experimental results demonstrate that antibody fragments may decrease the systemic exposure and toxicity of digoxin without affecting the peritoneal exposure, fraction unbound or disappearance rate. Systemic exposure and toxicity, therefore, have been dissociated from local drug exposure through the use of drug binding antibodies. These results suggest that drug toxicity may be prevented without affecting drug exposure at the active site.

TABLE 1

Digoxin tissue concentrations and Fu(p) for the antibody and control groups

Values are mean ± S.D.

	Time							
	5 min		15 min		30 min		60 min	
	Fab	Control	Fab	Control	Fab	Control	Fab	Control
	Tissue Concn of Digoxin (ng/g) (mean ± S.D.)							
Heart	24.8 ± 4.6**	77.5 ± 15.8	170.7 ± 93.4	277.0 ± 99.4	199.2 ± 44.5**	337.7 ± 34.3	137.2 ± 63.6*	286.0 ± 50.8
Muscle	6.1 ± 1.8*	24.3 ± 10.5	64.8 ± 67.8*	159.8 ± 23.6	30.2 ± 20.7***	238.6 ± 19.9	48.3 ± 52.6***	409.3 ± 21.0
Brain	3.9 ± 0.5**	10.2 ± 2.3	20.0 ± 5.0	15.2 ± 6.3	15.5 ± 1.3*	22.7 ± 4.4	11.5 ± 2.4***	23.7 ± 1.7
Fu(p)	0.80 ± 0.01	0.73 ± 0.004	0.83 ± 0.03	0.89 ± 0.19	0.86 ± 0.44	0.78 ± 0.16	0.65 ± 0.08	0.70 ± 0.16

* $P < 0.05$ compared to the control group; ** $P < 0.01$ compared to the control group; *** $P < 0.001$ compared to the control group.

The simulations conducted assumed a small value for the distribution clearance of Fab fragments between the peritoneal and central compartments. Unfortunately, we were unable to measure the rate of appearance of Fab in the peritoneal compartment to obtain an experimental estimate for this clearance value. However, the use of a small value was consistent with the experimental observation that the Fu(p) and the rate of digoxin disappearance from the peritoneal cavity were not different in the Fab fragment-treated group relative to the control group. If the distribution clearance of the Fab fragments had a larger value, then the unbound fraction of drug in the peritoneum and the rate of digoxin exiting the peritoneum would be expected to be altered.

The observed experimental results were predicted qualitatively through computer simulation. The use of a "physiological" model (Gerlowski and Jain, 1983) may allow for more accurate estimation of individual tissue exposures than the primitive "tissue cluster" model which was used for our simulations. However, the practical utility of such a model may be limited by its complexity. The model which we have presented may have general utility in describing drug disposition in the presence of binding substances. This model may be useful in screening drug candidates for the proposed antibody complexation approach, as well as for the development of optimal dosing strategies.

Although the utility of drug binding antibodies for the treatment of drug toxicity has been well documented (Smith *et al.*, 1976; Antman *et al.*, 1990; Brunn *et al.*, 1992), our results have demonstrated the potential utility of antidrug antibody fragments for the enhancement of drug site-specificity. It is possible that the proposed approach may be used as an adjunct in the treatment of cancers contained within the peritoneum. Systemic toxicity, particularly bone marrow depression, is often the dose-limiting factor in cancer chemotherapy. Optimization of i.p. drug delivery through antibody complexation may substantially shift the toxicity vs. dose curves of antineoplastic drugs administered into the peritoneum, thus allowing more drug to be given at acceptable levels of toxicity. Cure rates can be expected to follow the increase in administered dose, as most cancer chemotherapeutic agents are known to have a steep dose vs. response relationships.

There are, however, several concerns which must be addressed before the implementation of this type of therapy. First, the alteration of drug distribution which accompanies antibody-drug complexation may result in a potentiation of drug toxicities or the development of new drug toxicities in certain cases. For example, Faulstich *et al.* (1988) have shown an enhancement of the renal toxicity of α-amanitin (a mushroom toxin) when this toxin was coadministered with antiamanitin immunoglobulin G and Fab fragments. This toxicity is presumed to result from an increased delivery of the toxin (as the antibody-toxin complex) to protein-metabolizing cells of the kidney. The risk of redistributing systemic toxicity, rather than minimizing systemic toxicity, should be appreciated as a potential outcome of the proposed approach.

Additional important concerns are: 1) production and purification of antibodies directed against antineoplastic agents; 2) immunogenicity of the antibodies; and 3) cost of therapy. Further work on overcoming these formidable hurdles is required before the practicality of the proposed approach may be validated fully.

Acknowledgments

The authors gratefully thank Dr. Jerome Schentag for providing the Abbott TDx used in these experiments, Jeanmarie Acquard for expert technical assistance and Dr. James Crow of Burroughs Wellcome Co. for donation of the antidigoxin Fab used in these experiments.

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Journal of Immunological Methods, 59 (1983) 199–203
Elsevier Biomedical Press

Simplified Preparation of Rabbit Fab Fragments

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(Received 6 January 1982, accepted 21 December 1982)

Papain attached to solid-phase CH-Sepharose 4B was used to digest rabbit IgG. Protein A-Sepharose CL-4B was used to remove undigested IgG and Fc fragments. Pure Fab fragments free of IgG, Fc fragments and papain were readily obtained by this procedure with a yield of about 75%. Polyacrylamide gel electrophoresis of the Fab in the presence of sodium dodecyl sulphate gave a single band under both reducing and non-reducing conditions. The molecular weight of the Fab determined by sedimentation equilibrium was 49,200. Unlike the IgG, the Fab obtained did not form precipitin lines when used in immunoelectrophoresis.

Key words: *rabbit Fab — solid-phase papain — protein A*

Introduction

Fab fragments of IgG have been used in enzyme immunoassay (EIA) instead of IgG (Kato et al., 1976). EIAs of higher sensitivity have been claimed when Fab enzyme is used instead of IgG enzyme. The original method of Porter (1959) is a rather tedious procedure for preparing rabbit Fab fragments, although it is still widely used. Our requirement for such fragments arose from an investigation of the binding site(s) of snake neurotoxin at the neuromuscular junction. For this purpose a Fab enzyme conjugate was needed. We have used solid-phase techniques to simplify the preparation of rabbit Fab fragments.

Materials and Methods

Rabbit IgG

Immune and normal rabbit IgG were prepared by affinity chromatography of crude serum on protien A-Sepharose CL-4B (Pharmacia). The method used was that of Gosling (1976) as modified by Coulter et al. (1980). The immune serum had been raised against a neurotoxin (textilotoxin) isolated from the venom of the Australian brown snake, *Pseudonaja textilis*.

0022-1759/83/0000-0000/$03.00

Solid-phase papain

Five milligrams of papain (E.C. no. 3.4.22.2) type III, twice recrystallised, obtained from Sigma were reacted with 0.5 g of activated CH-Sepharose 4B (Pharmacia) according to the manufacturer's instructions.

Ninety-one per cent of the papain was covalently bound to the solid phase. Unbound papain was estimated by the Folin–Lowry procedure (Lowry et al., 1951).

The solid phase was stored as a 10% v/v suspension at 4°C in 0.05 M phosphate-buffered saline, pH 7.4 (PBS) containing 0.1% sodium azide.

Preparation of Fab

The procedure used was that described by Hudson and Hay (1976) except for the use of solid-phase papain and protein A to obtain Fab fragments.

The following reagents were added to 10 mg of rabbit IgG in 1 ml of PBS: 0.2 ml of 16 mg/ml cysteine hydrochloride, 0.2 ml of 5 mg/ml EDTA, sodium salt, 1 ml of solid-phase papain (equivalent to 1 mg), washed with 0.15 M phosphate, pH 7.0, to remove sodium azide.

The mixture was incubated with gentle stirring at 37°C for 4 h. The solid phase was sedimented by gentle centrifugation (1200 × g for 5 min) and the supernatant washed through a 4 ml protein A–Sepharose CL-4B column with 10 ml of PBS (pool A). Undigested IgG and Fc fragments were eluted from the column by successive washings with: 10 ml of PBS containing 0.1% Tween 20: this was discarded. Ten millilitres of PBS, discarded. Ten millilitres of 0.1 M glycine/HCl containing 1 M NaCl, pH 3. This was collected into 10 ml of PBS and adjusted to pH 7.4 with 1 M NaOH (pool B).

The column was finally stored in PBS containing 0.1% sodium azide.

Pools A and B were concentrated separately to about 5 ml by ultrafiltration over a PM-10 membrane in a model 52 cell (Amicon).

IgG and Fab concentrations were determined with values of $E^{1\%}_{1cm}$ = 14.0 and 14.5 respectively (Mandy and Nisonoff, 1963).

Immunoelectrophoresis (IEP)

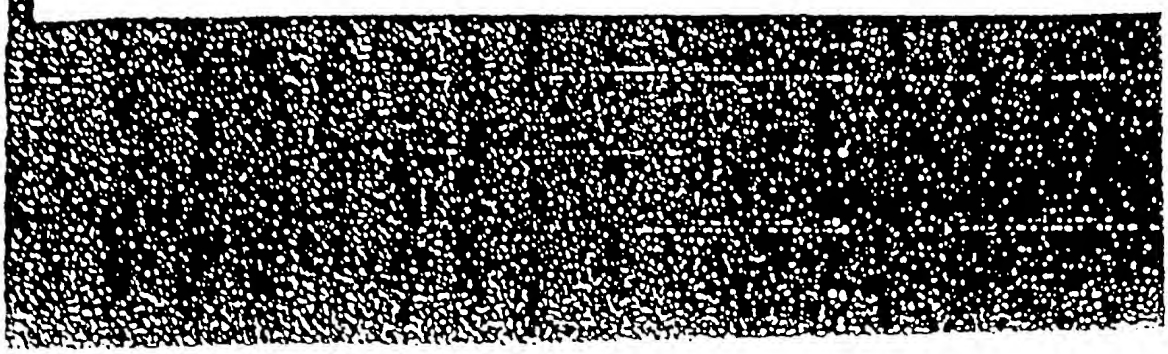
IEP was performed by the method of Scheidegger (1955). The antiserum used was rabbit anti-textilotoxin.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Weber and Osborn (1969) and Laemmli (1970). SDS was obtained from British Drug Houses as were acrylamide and methylenebisacrylamide. TEMED and 2-mercaptoethanol (2-ME) were from Eastman Organic Chem. Protein molecular weight standards were obtained from Pharmacia.

Analytical ultracentrifugation

The molecular weight determination was done by meniscus depletion in a Beckman model E analytical ultracentrifuge. The method of Yphantis (1964) was used. A $\bar{v}$ value of 0.73 cc/g was assumed for rabbit Fab.



Enzyme immunoassay (EIA)

EIA was used to determine the specific antibody titre after preparation of Fab from IgG. The EIA was as described previously (Coulter et al., 1981) for direct assay of snake venom. The wells of polystyrene microhaemagglutination plates (u-wells, Cooke Lab. Prod.) were coated with textilotoxin. The following procedure was used.

Plates were floated in a water bath at 37°C for 2 h after adding 0.1 ml of 1 μg/ml textilotoxin in 0.05 M sodium carbonate buffer, pH 9.6, to the wells. The plates were washed and incubations performed as previously described (Coulter et al., 1981). The relative ability of rabbit IgG anti-textilotoxin and Fab anti-textilotoxin to block the uptake of rabbit IgG anti-textilotoxin–horse radish peroxidase (HRPO) conjugate by the polystyrene bound textilotoxin was determined in the following way. Serial dilutions of IgG and Fab were incubated in wells coated with textilotoxin for 30 min at 37°C. After washing and subsequent reaction with conjugate the degree to which IgG and Fab had blocked conjugate uptake was determined by intensity of colour development on addition of substrate to the wells.

Mouse protection assays

IgG and Fab anti-textilotoxin were tested for their ability to neutralise the lethal effects of textilotoxin in mice. Eighteen to 21 g Swiss mice (CSL strain) were used in the assay. Four mice were used at each dose level and all injections were given intravenously. Four LD_{50} of textilotoxin were incubated with serial dilutions of immune IgG and Fab. All doses were given in a volume of 0.2 ml. A 0.1% solution of bovine serum albumin in 0.85% NaCl was used as diluent; this reduces non-specific adsorption of textilotoxin (Broad et al., 1979).

IgG and Fab prepared from normal rabbit IgG were used as controls.

Ion exchange chromatography of Fab prepared by the solid-phase papain/protein A procedure

Fab prepared by the solid-phase papain/protein A procedure was subjected to ion exchange chromatography. The method of Porter (1959) as modified by Hudson and Hay (1976) was used.

Results and Discussion

Fab fragments prepared by the solid-phase papain/protein A procedure gave a single diffuse band on SDS-PAGE run under non-reducing conditions. A single band of M_r 27,000 was obtained on SDS-PAGE run under reducing conditions (Fig. 1). Rabbit Fab and Fc fragments have M_rs of about 50,000 (Marler et al., 1964). No IgG was detectable by SDS-PAGE in the Fab preparation.

A M_r of 49,200 was obtained by sedimentation equilibrium. The M_r was constant through most of the solution column, with a slight increase towards the base of the cell.

Dialysis of the Fab preparation against a nominal 10,000 M_r cut-off membrane ensures removal of low M_r products. Apart from the Fab band, no other high or low

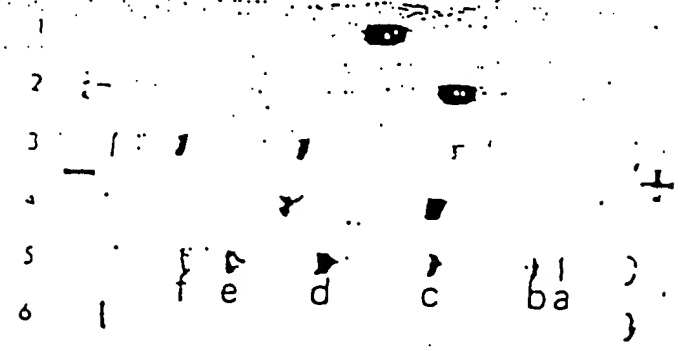


Fig. 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Laemmli, 1970) of rabbit IgG and its fragments resulting from solid-phase papain digestion. Essentially the same results were obtained with the Weber and Osborn (1969) gel system (not shown). Gel 1: Non-protein A bound material after solid-phase papain digestion, i.e., Fab run under non-reducing conditions. Gel 2: Fab prepared by the solid-phase papain procedure. Sample reacted with 2-ME. Gel 3: protein A bound components after solid-phase papain digestion. These were eluted at low pH. Gel run under non-reducing conditions. Gel 4: sample as for gel 3, treated with 2-ME. Gel 5: protein M_r standards: a, α-lactalbumin, 14,400; b, trypsin inhibitor, 20,100; c, carbonic anhydrase, 30,000; d, ovalbumin, 43,000; e, albumin, 67,000; f, phosphorylase b, 94,000. Gel 6: blank gel.

M_r products were visible by SDS-PAGE.

When the Fab preparation was fractionated by ion exchange, 94% of the starting material was recovered. Sixty-five per cent of the starting material was eluted with the equilibrating buffer (0.01 M sodium acetate, pH 5.5) and 29% was eluted before a sodium acetate concentration of 0.2 M was reached. No other material was eluted, even when 1 M sodium acetate, pH 5.5, was used.

When the anti-textilotoxin titre of the Fab preparation was compared with that of IgG by EIA, the Fab concentration which yielded 50% of full colour in the assay was 4.5 μg/ml. The IgG concentration which gave the same colour was 6.2 μg/ml. In terms of anti-textilotoxin activity in the EIA, when their relative M_rs are considered, the Fab and IgG were practically equivalent. The final yield of Fab was always about 75% of that theoretically possible, i.e., 5 mg Fab from 10 mg of IgG.

The neutralisation tests performed in mice showed that on a weight basis the IgG and Fab anti-textilotoxin preparations were equivalent in neutralising ability, i.e., a loss of approximately 30% of specific antibody activity.

When equivalent concentrations of Fab and IgG anti-textilotoxin were tested by IEP against textilotoxin, precipitin lines were obtained with IgG but not with the Fab preparation.

These results demonstrate that Fab fragments can be obtained from rabbit IgG with losses of 20–30% of initial IgG antibody activity. The steps involved in the procedure are relatively simple. The solid-phase papain is not difficult to prepare;

the reaction with IgG is straightforward as in the protein A purification of the Fab. The procedure represents a marked improvement in terms of simplicity and time saving over the ion exchange procedure for the preparation of rabbit Fab fragments.

Note Added in Proof

Since completing this paper an important article came to our attention describing the same experimental procedure:

Goding, J.W., 1978. J. Immunol. Methods 20, 241.

Acknowledgements

The assistance of Mr. Allen Broad and Miss Wendy Cowling with the in vivo assays was greatly appreciated. Mr. John Cox and Dr. Struan Sutherland were most helpful in discussion. We thank Dr. Frank Woods of the Protein Chemistry Division of the CSIRO for the analytical ultracentrifugation.

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Library of Congress Cataloging-in-Publication Data

Casarett and Doull's toxicology : the basic science of poisons /
editor, Curtis D. Klaassen, editors emeriti, Mary O. Amdur, John
Doull.—5th ed.
p. cm.
Includes bibliographical references and index.
ISBN 0-07-105476-6
1. Toxicology. I. Casarett, Louis J. II. Klaassen, Curtis D.
III. Amdur, Mary O. IV. Doull, John, date.
[DNLM: 1. Poisoning. 2. Poisons QV 600 C335 1995]
RA 1211.C296 1995
615.9—dc20
DNLM/DLC
for Library of Congress 95-22362

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[DNLM: 1. Poisoning. 2. Poisons. QV 600 C335 1995]
RA 1211.C298 1995
615.9—dc20
DNLM/DLC
for Library of Congress 95-22362

CHAPTER 26

TOXIC EFFECTS OF ANIMAL TOXINS

Findlay E. Russell

Venomous or poisonous animals are found in all the animal classes, including the birds. For the most part, they are widely distributed throughout the animal kingdom from the unicellular protistan *Alexandrium (Gonyaulax)* to certain chordates, including the platypus and the short-tailed shrew. Venomous marine animals are found in almost all seas and oceans. Although there are no exact figures on the numbers of such animals, there are approximately 1200 species of venomous or poisonous marine animals (Russell, 1984a), the number of venomous arthropods is countless, and there are about 400 species of snakes considered dangerous to humans.

The term "venomous animal" usually is applied to creatures that are capable of producing a poison in a highly developed secretory gland or group of cells and can deliver that toxin during a biting or stinging act. "Poisonous animals," by contrast, are generally regarded to be those whose tissues, either in part or in their entirety, are toxic. These animals have no mechanism or structure for the delivery of their poisons. Poisoning in these forms usually takes place through ingestion (Russell, 1965).

A venom may have one or several functions in an animal's armament. It may play a role in offense, as in the capture and digestion of food, or may contribute to the animal's defense, as in protection against predators or aggressors. It also may serve both functions. The principal biological property of the venom of a snake, however, is its food-securing potential. In this respect, venom is a superior modification to speed, size, strength, and better concealment as well as other characteristics seen in nonvenomous snakes. In addition, venom plays a role in the digestion of the prey. Finally, venom can play a role in a snake's defensive posture, as in spitting cobras and ringhals, and in kills or underkills in a defensive situation (Russell, 1984b).

The black widow spider and many other species of spiders use venom to paralyze their prey before extracting hemolymph and body fluids. It appears that the venom is not designed primarily to kill the prey, only to immobilize it. The venom apparatus of the stingray is used for defense. It is not employed in getting food, and for the most part its defensive use appears to have been spent eons ago. Lionfishes, stonefishes, and weeverfishes also use their venomous spines for defense. Scorpions, by contrast, can use their venom for both offense and defense.

Most venoms used in an offensive posture are associated with the oral pole of the animal, obviously the most functional place for their delivery. Defensively designed venoms usually are associated with the aboral pole, as in stingrays, or with dermal tissues, as in scorpionfishes and certain other fishes (Russell, 1965; Halstead, 1965).

In poisonous animals the poison or toxin may play a small role, if any, in the animal's offensive or defensive activities. The poison may be a product or by-product of metabolism or a product passed along in the food chain. In the case of Tetraodontidae, the responsible toxic organisms may be the Vibrionaceae and perhaps other bacteria. In ciguatera fish poisoning, the toxic organism, a dinoflagellate, is ingested by herbivores and then by carnivores. Ciguateric fishes in human poisonings have fed on smaller toxic fishes or other toxic marine animals, which in turn have ingested *Gambierdiscus* spp. or other toxic organisms. At each step in the feeding process, more toxin is accumulated; thus, while poisoning in humans may not result from eating the smaller toxic fishes or marine organisms, by the time a large grouper, barracuda, snapper, or other toxic fish that has fed on smaller toxic fishes is eaten, poisoning occurs. This sequence of events is known as the food-chain phenomenon, as described by Halstead (1965).

PROPERTIES OF ANIMAL TOXINS

As one might expect from the various uses to which animals put their poisons, these toxins vary considerably in their chemistry and toxicology. Venoms, for instance, may be composed of proteins of both high and low molecular weight, including polypeptides and enzymes. They also may be amines, lipids, steroids, aminopolysaccharides, quinones, 5-hydroxytryptamine (5-HT), glycosides, or other substances. The biological properties of snake venoms have been reviewed by Zeller (1948), Russell (1967, 1983), Dowling and associates (1968), Minton and Minton (1969), Elliott (1978), Tu (1977), Lee (1979), and Habermehl (1981). With respect to the venoms of spiders, the text of Maretić and Lebez (1979) provides a fine description and ample references. Keegan's (1980) work on the scorpions is a basic text that provides a good overview of these arthropods. The toxins of marine animals have been thoroughly described by Halstead (1965, 1978) and Russell (1965, 1984a). The series of texts by Scheuer (1973, 1978) provides additional data on some marine animal toxins, as does the excellent work of Southcott (1979) and Sutherland (1983). Readers will find the book by Hashimoto (1979) a useful source of data on the biochemistry of marine toxins. Those specifically interested in ciguatera poisoning will find the recent contribution of the *Memoirs of the Queensland Museum* an excellent review.

One of the unfortunate facts in the study of the chemistry, pharmacology, and toxicology of venoms is that their structure and function are most easily researched by taking the venoms apart. This has two shortcomings: First, a destructive process is used in an attempt to understand an expedient and integrative one; second, the essential quality of the venom may be destroyed before a suitable acquaintance with it has been made. Often, the process of examination becomes so exacting that

the end is lost sight of in the preoccupation with the means: in some cases the means may become a substitute for the end.

Another shortcoming in the study of venoms has been the naive and oversimplified habit of classifying the whole poison or even its component parts as "neurotoxins," "cardiotoxins," "hemotoxins," "myotoxins," and other inexact synonyms. Most venoms probably exert their effects on almost all cells and tissues, and their pharmacological properties are determined by the amount of a specific biologically active component that accumulates at an activity site where it is capable of producing a change. That change probably has a common chemical basis in most tissues, specific not only to the component but also to the alteration in ion exchange it may cause at a cell or tissue site. Of course, most venoms have a more particular effect on one or several tissue sites, but recent experimental work has demonstrated the wide scope of the toxicological effects a venom or venom fraction can precipitate.

A clinician must never ignore any symptom or sign in a patient or minimize any manifestation on the naive assumption that the venom has to be a "neurotoxin," "cardiotoxin," "hemotoxin," or "myotoxin" with its activity limited to one organ or system. While the patient may have respiratory distress from a "neurotoxic venom," he or she can also have changes in cardiac dynamics or vascular permeability, and these changes can become far more life-threatening, particularly if the physician centers his or her attention and therapy on the so-called neurotoxic activity of the venom (an effect that often can be treated adequately with simple positive-pressure respiration). The physician must guard his or her knowledge and experience zealously and be aware of the limits of application of pharmacological data that are based on animal experimentation.

Venoms may have important properties aside from the specific activities of their component parts. Important synergisms that are not obvious from the study of individual fractions may become apparent in studies of the activity of the whole venom. In addition, the whole venom may precipitate autopharmacological reactions that are not produced by individual fractions. Finally, the problem of the formation of metabolites in an envenomated organism has not been explored in a definitive manner, and this can be an important consideration in clinical cases.

The action of a venom or venom component is dependent on a number of variables, including its route of administration, absorption, distribution, passage across a succession of membranes, accumulation and action at a receptor site, and metabolism and excretion (Russell, 1980a). All these factors play a role in determining the action of the toxin. During the past two decades it has become increasingly clear that there are significant variations in the roles of these factors in different venoms and different species of animals. In some cases the variations in different animals are more important than the difference usually attributed solely to the weight of the animal. Studies carried out in pigs, opossums, certain species of rats, and other animals purport to show that these animals are more "immune" to a toxin than are mice. Such investigations do not take into account the dependent physiological variables involved in the availability and processing of a toxin in different kinds of animals, influences that are not related to any principle of immunity. It is a fallacious assumption to treat the LD_{50} of mice and that of the opossum or another animal as direct products of the differences in their weights. In this respect, the toxicologist must always be concerned with the question of whether a particular difference between animals is caused by variables in the effectiveness of the toxin at the receptor site or in its absorption, distribution, metabolism, or excretion. The fate of a venom or venom fraction, as its activities are spent in the animal, has been discussed elsewhere (Russell, 1980a, 1980b, 1983).

ANTIVENOMS (ANTIVENINS)

Because of their protein composition, many toxins produce an antibody response; this response is essential in producing antisera. An antivenom consists of venom-specific antiserum or antibodies concentrated from immune serum to the venom. Antisera contain neutralizing antibodies: one antigen (monovalent) or several antigens (polyvalent). Animals immunized with venom develop a variety of antibodies to the many antigens in the venom. The serum is harvested, partially or fully purified, and further processed before being administered to the patient. The antibodies bind to the venom molecules, rendering them ineffective. Antivenoms have been produced against most medically important snake, spider, scorpion, and marine toxins.

Antivenoms are available in several forms: intact IgG antibodies or fragments of IgG such as $F(ab)_2$ and Fab. They are prepared through $AmSO_4$ or Na_2SO_4 precipitation, pepsin or papain digestion, and other procedures, among which the elimination of the Fc, or complement-binding and complement-sensitizing fraction, is one of the most important. The molecular weight of the intact IgG is about 150,000, whereas that of Fab is approximately 50,000.

The molecular size of IgG prevents its renal excretion and produces a volume of distribution much smaller than that of Fab. The elimination half-life of IgG in the blood is approximately 50 h. Its ultimate fate is not known. Most IgG probably is taken up by the reticuloendothelial system and degraded with the antigen attached. Fab fragments have an elimination half-life of about 17 h and are small enough to permit renal excretion.

Since all antivenom products are produced through the immunization of animals, this increases the possibility of hypersensitivity. Type I (immediate) hypersensitivity reactions are caused by antigen cross-linking of endogenous IgE bound to mast cells and basophils. Binding of antigen by a mast cell may cause the release of histamine and other mediators, producing an anaphylactic reaction. Once initiated, anaphylaxis may continue despite discontinuation of antivenin administration. An additional concern is an *anaphylactoid* reaction. This is a term for a syndrome resembling an anaphylactic reaction; its etiology is unknown but appears to be associated with aggregated protein in the antiserum. Protein aggregates may activate the complement cascade, producing an anaphylactic-like syndrome. An important difference between anaphylactic and anaphylactoid reactions is that anaphylactoid reactions are dose-dependent and may be halted by removing the antigen. Type III hypersensitivity (serum sickness) may develop several days after antivenom administration. In these cases, antigen-antibody complexes are deposited in different areas of the body, often producing inflammatory responses in the skin, joints, kidneys, and other tissues. Fortunately, these reactions

Table 26–1
Some Medically Important Snakes of the World (*Continued*)

SCIENTIFIC AND COMMON NAMES	DISTRIBUTION
Naja nivea—Cape or yellow cobra	Nambia, Botswana south to the Cape
Ophiophagus hannah—king cobra	Indian subcontinent, China and Philippines
Walterinnesia aegyptia—desert blacksnake or desert cobra	Egypt to Iran
Kraits and mambas	
Bungarus caeruleus—Indian or blue krait	India, Pakistan, Sri Lanka, Bangladesh
Bungarus candidus—Malayan krait	Thailand, Malaysia, Indonesia
Bungarus multicinctus—many-banded krait	Southern China to Hainan, Taiwan
Dendroaspis polylepis—black mamba	Ethiopia and Somalia to Angola, Zambia, Nambia, southwest Africa
Australian elapids	
Acanthophis antarcticus—common death adder	Most of Australia, Moluccas, New Guinea
Notechis scutatus—tiger snake	Southeastern Australia
Oxyuranus scutellatus—Taipan	Northern coastal Australia, parts of New Guinea
Pseudechis australis—mulga	Most of Australia except southeast and southern coast, New Guinea
Pseudonaja nuchalis—western brown snake	Most of Australia except east and southeast coast
Pseudonaja textilis—eastern brown snake	Eastern Australia

Note: The common names in this table are those generally employed as literature identifications for the snakes. However, these names may not be the ones used by people in the specific area where the snake abounds.

Thrombinlike enzymes have been used clinically and in animals for therapeutic and investigative studies. In experimentally induced venous thrombosis in dogs, treatment with ancrod before the formation of the thrombus prevented thrombosis and ensured vessel patency. However, ancrod had no thrombolytic effect when administered after thrombus formation. Trials of ancrod versus heparin and ancrod versus streptokinase in the treatment of deep venous thromboses of the lower leg have been conducted. It appears that neither heparin nor ancrod has a significant effect on thrombus resolution, whereas streptokinase produces more lysis of thrombi than does ancrod. Crotalase has been employed to evaluate the role of fibrin deposition in burns in animals (Bajwa and Markland, 1976). The role of fibrin deposition has been evaluated in tumor metastasis, in which fibrinogen is removed by treatment with ancrod or batroxobin. Ancrod also has been used to prevent the deposition of fibrin on prosthetic heart valves implanted in calves (Russell, 1980b, 1983).

Collagenase is a specific kind of proteinase that digests collagen. This activity has been demonstrated in the venoms of a number of species of crotalids and viperids. The venom of *Crotalus atrox* digests mesenteric collagen fibers but not protein. EDTA inhibits the collagenolytic effect but not the arginine esterase effect.

Hyaluronidase catalyzes the cleavage of internal glycoside bonds in certain acid mucopolysaccharides. This results in a decrease in the viscosity of connective tissues. The breakdown in the hyaluronic barrier allows other fractions of venom to penetrate the tissues. The enzyme is thought to be related to the extent of edema produced by the whole venom, but the degree to which it contributes to clinical swelling and edema is not known. The enzyme also has been referred to as the "spreading factor."

Phospholipase enzymes are widely distributed throughout animals, plants, and bacteria. Snake venoms are the richest sources of phospholipase A_2 (PLA_2) enzymes. PLA_2 catalyzes the hydrolysis of the fatty acid ester at the 2-position of diacyl phosphatides, forming lysophosphatides and fatty acids, primarily unsaturated. The complete amino acid sequences of over 50 snake venom PLA_2 enzymes have been determined. The enzyme is widely distributed in the venoms of elapids, vipers, crotalids, sea snakes, and several colubrids. Although the sequences of these enzymes are homologous and their enzymatic active sites are identical, they differ widely in their lethal indexes and pharmacological properties. For example, taipoxin, a PLA_2 enzyme from the venom of the Australian elapid *Oxyuranus scutellatus* has an IV LD_{50} in mice of 2 μg/kg, whereas the neutral PLA_2 from *Naja nigricollis* has an LD_{50} of 10,200 μg/kg, even though *N. nigricollis* PLA_2 is enzymatically more active.

Recent studies have shown that PLA_2 enzymes can exert their pharmacological effects by different mechanisms: hydrolysis of membrane phospholipids, liberation of pharmacologically active products, and effects independent of enzymatic action. Similarly, snake venom PLA_2 enzymes can be

Table 26–2
Enzymes of Snake Venoms

Proteolytic enzymes	Phosphomonoesterase
Arginine ester hydrolase	Phosphodiesterase
Thrombinlike enzyme	Acetylcholinesterase
Collagenase	RNase
Hyaluronidase	DNase
Phospholipase A_2(A)	5′-Nucleotidase
Phospholipase B	NAD-nucleotidase
Phospholipase C	L-Amino acid oxidase
Lactate dehydrogenase	

Source: Russell (1983).

effective whereas the latter was not. Clinical trials of Brazilian, Ecuadorian and possibly Colombian antivenoms are planned in the Amazon region of Ecuador in the near future.

Theakston, R. D. G. and Reid, H. A. (1983) *Bull. WHO* 61, 949–956.

Comparison of $F(ab')_2$ and Fab efficiency on plasma extravasation induced by Viper aspis *venom.* M. Sorkine,[1,2] B. Saliou[1] and C. Bon[1] ([1]Unité des Venins, Institut Pasteur 25, Rue du Dr Roux, 75724 Paris Cedex 15, France; and [2]S.A.R., Hôpital Henri Mondor, Créteil 94000, France).

Envenomation caused by European vipers associates local signs, essentially oedema and systemic manifestations. Extensive oedema produces pain and inability to use the affected limb, and is a major factor of hypovolemia. Since symptomatic treatment failed to prevent this oedema, the effect of antivenom on plasma extravasation, the first step of oedema formation, was examined. The purpose of the study was to compare in a mouse model the effect of $F(ab')_2$ (equine) and Fab (equine and ovine) on capillary permeability increase (CPI) induced by *Vipera aspis aspis* venom. $F(ab')_2$ (ID_{50} 2 mg/kg) and Fab (ID_{50} 2.5 mg/kg) reduced considerably CPI when mixed with venom prior to intradermal injection. When fragments were intravenously injected before intradermal administration of the venom, a larger amount of fragments was necessary, Fab being five times more effective than $F(ab')_2$ (ID_{50} 105 mg/kg compared to ID_{50} 520 mg/kg). Furthermore, immunoglobulins injected after the venom $F(ab')_2$ were ineffective, while Fab has a residual effect (ID_{50} 235 mg/kg). No difference was observed on the efficiency of ovine and equine Fab. These data showed firstly that the *in vitro* neutralization of the venom by immunoglobulin fragments does not reflect their *in vivo* efficiency. Secondly, Fab was considerably more effective than $F(ab')_2$ in reducing CPI-induced by venom. One explanation is the different kinetics of these fragments. The smaller size of Fab results in faster diffusion and a greater volume of distribution.

Molecular structure and action mechanism of the specific crotoxin inhibitor from Crotalus durissus terrificus *serum.* J. Perales,[1] C. Villela,[1] G. Domont,[1] V. Choumet,[2] B. Saliou,[2] H. Moussatché[1], C. Bon[2] and G. Faure[2] ([1]Departamento de Fisiologia e Farmacodinâmica, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil; and [2]Unité des Venins, Institut Pasteur, Paris, France).

An antivenom protein component that specifically neutralizes crotoxin, the main lethal component of rattlesnak